

A Trio–Rac1–Pak1 signalling axis drives invadopodia disassembly

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Rho family GTPases control cell migration and participate in the regulation of cancer metastasis. Invadopodia, associated with invasive tumour cells, are crucial for cellular invasion and metastasis. To study Rac1 GTPase in invadopodia dynamics, we developed a genetically encoded, single-chain Rac1 fluorescence resonance energy (FRET) transfer biosensor. The biosensor shows Rac1 activity exclusion from the core of invadopodia, and higher activity when invadopodia disappear, suggesting that reduced Rac1 activity is necessary for their stability, and Rac1 activation is involved in disassembly. Photoactivating Rac1 at invadopodia confirmed this previously unknown Rac1 function. We describe here an invadopodia disassembly model, where a signalling axis involving TrioGEF, Rac1, Pak1, and phosphorylation of cortactin, causes invadopodia dissolution. This mechanism is critical for the proper turnover of invasive structures during tumour cell invasion, where a balance of proteolytic activity and locomotory protrusions must be carefully coordinated to achieve a maximally invasive phenotype.

Tumour invasion requires orchestration of actin-based protrusions capable of extracellular matrix (ECM) degradation and cell locomotion^{1–3}. On epithelial to mesenchymal transformation⁴, tumours gain the ability to invade by protruding invadopodia, characterized by their ability to localize matrix metalloproteinases important for the proteolytic digestion of ECM (refs 5,6). In breast carcinomas, the ability of cells to form invadopodia and their invasive potential are directly correlated⁷; thus, understanding the molecular mechanisms regulating invadopodia functions is critical.

Cells assemble and protrude invadopodia during invasion⁸ and several members of the p21 Rho family of small GTPases, including Cdc42, RhoA and RhoC, are involved^{9,10}. These GTPases cycle between a GTP-loaded 'ON' versus a GDP-loaded 'OFF' state¹¹. Regulators controlling these states include guanine nucleotide exchange factors (GEF), GTPase activating proteins (GAP), and guanine nucleotide dissociation inhibitors¹² (GDI). Cdc42 is crucial for invadopodia formation by activating neuronal Wiskott–Aldrich Syndrome protein (N-WASP), on which other invadopodia core proteins are assembled¹³. RhoC regulates invadopodia integrity by confining actin protrusion within the invadopodium¹⁰. RhoA is involved in the delivery of vesicles containing membrane type 1 matrix metalloproteinases to degradation sites^{10,14}, and other functions associated with the actin cytoskeleton⁹. The mechanism by which these GTPases regulate their respective downstream functions at the invadopodia is spatially distinct¹⁰. This suggests

that the spatiotemporal dynamics of Rho GTPase activations at or surrounding invadopodia are critical, yet not much is known about such dynamics of other Rho isoforms, specifically Rac1. Previous studies indicated that Rac1 overexpression and hyperactivity lead to aberrant cell motility and metastatic phenotypes^{15–21}. Rac1 was necessary for invasive protrusions in human melanomas²², and MCF10A breast epithelial cells required Rac1 for TGF- β -dependent matrix degradation²³. Rac1 was also required for invasion in Ras-transformed melanoblasts²⁴. Although Rac1 seems to be required for pro-invasive functions in these cases, there is not yet a clear study of Rac1 during invadopodia functions at subcellular scales. A number of studies have begun to address this question^{22,23,25}, and have shown evidence that Rac1 activation may drive invadopodia. However, this has never been directly observed, only indirectly inferred on the basis of traditional experimental methods. Invadopodia are highly regulated and transient subcellular structures, and Rac1 has equally fine spatiotemporal activation dynamics within cells²⁶, making it difficult to accurately study using more conventional approaches.

To address this problem, we developed a FRET-based biosensor for Rac1. The biosensor enables direct visualization of Rac1 activities at subcellular resolution and on a timescale of seconds, while maintaining a single-chain structure and correct isoprenylation. Using this biosensor in combination with the focal photo-uncaging of Rac1 (ref. 27), we report a mechanism by which invasive breast carcinomas

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disassemble their invadopodia through Trio–Rac1 activation, through cortactin phosphorylation by p21 activated kinase 1 (Pak1). Here we report the involvement of Rac1 in invadopodia turnover, which could be essential in proper regulation of invasive protrusions during invasion and metastasis.

RESULTS

Rac1 restricts matrix-degrading invadopodia activity

To determine the requirement for Rac1 in invadopodia formation and function, we first silenced Rac1 in MTLn3 rat mammary adenocarcinoma cells²⁸ and assayed for matrix degradation activity¹⁰. On Rac1 depletion²⁸ (Fig. 1a), degradation of extracellular matrix is markedly increased compared with control short interfering RNA (siRNA)-treated cells (Fig. 1b,c), suggesting that Rac1 controls invadopodia activity. This was recapitulated when Rac1 inhibitor NSC23766 was used (Fig. 1c,d). This is recapitulated in human MDA-MB231 and BT549 metastatic cell lines^{29–31}, but not in the MCF10A normal human breast epithelial cell line³² (Supplementary Fig. 1a–g). MTLn3 express Rac1 and Rac3, but not Rac2 (Fig. 1e). Rac1 RNA-mediated interference (RNAi) did not affect Rac3 expression levels (Fig. 1e), and multiple single siRNA oligonucleotides also resulted in the observed phenotype (Supplementary Fig. 1h). Enhanced degradation from Rac1 knockdown was probably not due to lack of cell motion, as the relative invadopodia lifetimes in these conditions were greatly increased, but the average number of invadopodia per cell at any one point in time remained the same (Fig. 1d and Supplementary Video 1). These results suggest that Rac1 activity seems to restrict invadopodia lifetime as well as matrix degrading activity.

A single-chain Rac1 biosensor

Biochemical approaches cannot be used for observing Rac1 activity at invadopodia; we produced a genetically encoded single-chain Rac1 biosensor. A full-length Rac1 was placed at the carboxy terminus of the biosensor to maintain the hypervariable region and the correct lipid modification motif, important for plasma membrane and GDI interactions, similar in design to the previous RhoA biosensor³³ (Fig. 2a,b). GDI interaction is the main difference to the previous-generation Rac1 biosensor, reflecting the regulatory cycle of Rac1 in sensor readouts^{34,35}.

The sensor consists of a monomeric Cerulean, two tandem p21-binding domains (PBD) of Pak1, separated by a structurally optimized 14 amino-acid linker chain, monomeric Venus, and full-length Rac1. The tandem PBD allows for autoinhibitory mechanism of Pak1 by maintaining the autoregulatory moiety (amino acids 70–149; ref. 36). The second PBD contains H83D–H86D GTPase-binding mutations to prevent spurious interactions with other GTPases when the biosensor is activated and the autoinhibition is relieved (Fig. 2c).

As the size of the biosensor precluded *in vitro* purification, we overexpressed it in HEK293 cells and analysed the fluorescence spectra of mutant versions of the biosensor as previously described³³. Compared with wild-type (WT), the constitutively active (G12V and Q61L) mutants had a higher emission ratio (FRET/CFP), and the inactive mutants (T17N and T35S) had a reduced emission ratio. Co-expression of threefold excess GDI produced a reduced FRET ratio as expected in WT and G12V mutant versions of the biosensor (Fig. 2d,e).

When co-transfected with excess GDI, GEFs that are known to activate Rac1 (ref. 37) rescued Rac1 activity, but non-Rac1 GEFs did not rescue activity (Fig. 2f). Similarly, p50RhoGAP inhibited Rac1 activity, but the control Rap1GAP did not (Fig. 2f). These data show that the biosensor behaves accurately and is regulated by upstream regulators. The overexpression of G12V and T17N versions of the biosensor in MEFs shows that the in-cell difference in the FRET ratio based on imaging is approximately 40%, similar to the fluorometric analysis (Fig. 2g). In versions of the biosensor where both PBDs were mutated (H83D–H86D), FRET was reduced to dominant-negative and GDI-inhibited levels (Fig. 2h).

Rac1 activity is excluded from invadopodia

We produced an MTLn3 cell line stably incorporating the Rac1 biosensor under tet-OFF regulation to limit the biosensor expression to $\leq 20\%$ of endogenous Rac1 (Fig. 2i; refs 33,38). We stimulated cells with EGF, triggering invadopodia precursor formation⁸ and observed Rac1 activity during early events. After stimulation, structures containing cortactin fluorescence appeared and remained throughout the 10-min duration of imaging, accompanied by a pronounced depletion of Rac1 activity within the invadopodium core (Fig. 3a,b and Supplementary Video 2). This exclusion of Rac1 activity was not due to loss of Rac1 from the invadopodia, as endogenous Rac1 or CFP–Rac1 is uniformly distributed (Fig. 3c and Supplementary Fig. 2a). A similar reduction in Rac1 activity was observed in fixed cells using Cy5-dye-labelled cortactin (Fig. 3d). MDA-MB-231 cells also show reduced Rac1 activity at invadopodia (Fig. 3e,f). Accumulated cortactin puncta are non-vesicular³⁹ and degrade matrix (Supplementary Fig. 2b,c).

We examined how Rac1 is involved in invadopodia maintenance by observing Rac1 activity in steady-state invadopodia over a period of 3 h. We began imaging pre-formed invadopodia and examined Rac1 activity throughout their lifetimes. We observed that when a stable invadopodium is present, Rac1 activity inside the invadopodium is significantly lower when compared with the region just outside (Fig. 3b), until the invadopodium disappears (Fig. 3g and Supplementary Video 3). At this point Rac1 activity is elevated (Fig. 3h and Supplementary Fig. 2d). This suggests that Rac1 activation could be associated with disassembly of invadopodia. As a control we compared this Rac1 activity profile to RhoA activity³³. As shown previously for EGF-induced invadopodia¹⁰, steady-state RhoA activity also showed no significant changes (Supplementary Fig. 2e).

Focal activation of Rac1 disassembles invadopodia

To determine whether Rac1 activation triggers invadopodia disassembly, we used photoactivatable Rac1 (PA-Rac1; ref. 27) to activate Rac1 at pre-formed invadopodia and observe how it influences their dynamics. After photoactivation using 1-s pulses of 457 nm light every 30 s to MTLn3 cells expressing PA-Rac1 and two invadopodia markers (mTagRFP–cortactin and GFP–Tks5; ref. 40), the invadopodium that was photoactivated disappears within 6 min of the start of the irradiation, at the same time the uncaged Rac1 produces a lamellipodium protrusion next to the photoactivated region (Fig. 4a,c and Supplementary Video 4). Approximately 80% of invadopodia analysed disappear on focal photoactivation of Rac1 (Fig. 4d). We recapitulated this effect in MDA-MB-231 cells (data not shown). Approximately 39% (18/46) of the time there are multiple invadopodia present, and

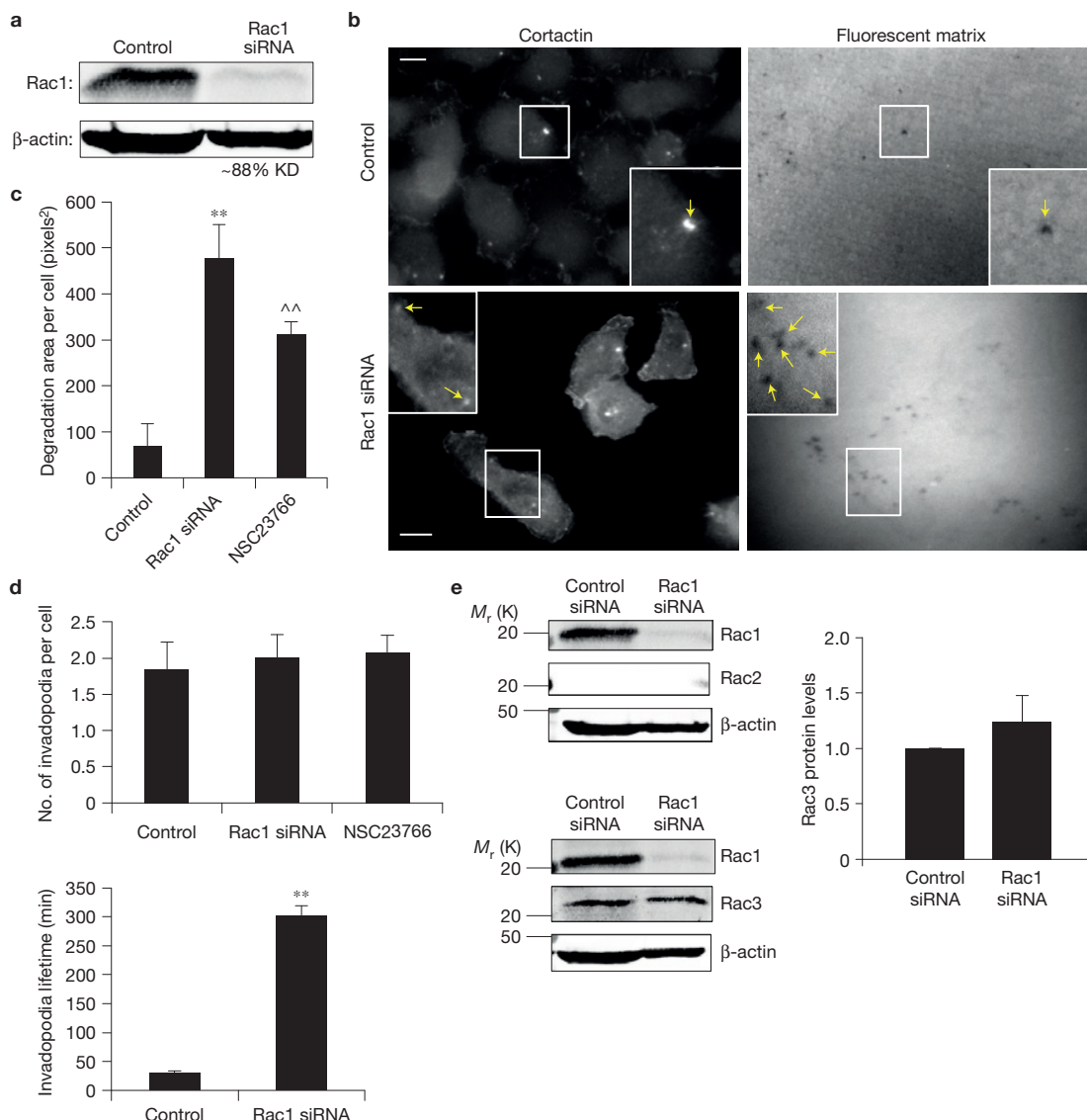


Figure 1 Rac1 depletion increases invadopodia degradation activity. **(a)** Western blot of cell lysates of control and Rac1-siRNA-treated MTLn3 cells, blotted for Rac1 and β -actin. A representative image is shown from 3 blots. **(b)** MTLn3 cells transfected with control or Rac1 siRNA and plated on Alexa-405-conjugated gelatin overnight. Arrows point to invadopodia and sites of degradation. Scale bars, 10 μ m. Representative image sets are shown from 50 image sets each for the control and Rac1 siRNA. **(c)** Quantification of mean degradation area per cell from **b**, including Rac1 inhibitor NSC23766 treatment at 100 μ M. $n=60$ fields for each condition, pooled from 5 independent experiments; error bars are s.e.m. Student's t -test was used. **(d)** Quantification of average number of invadopodia per cell, and mean invadopodia lifetime from time-lapse movies over a period of 6 h. Invadopodia lifetime was quantified as the time in minutes from the appearance to the disappearance of RFP-cortactin

and Tks5-GFP co-localization spots. $n=9$ invadopodia for each condition, pooled from 3 independent experiments; error bars are s.e.m. Student's t -test was used. $P=0.418134$ (number of invadopodia per cell, control versus Rac1 siRNA), $P=0.115958$ (number of invadopodia per cell, control versus NSC23766), $**P=6.531 \times 10^{-27}$ (invadopodia lifetime). **(e)** MTLn3 cells contain Rac1 and Rac3, but not Rac2, and Rac1 siRNA knockdown does not alter the levels of Rac3. Western blot of MTLn3 cells treated with control or Rac1 siRNA and blotted for Rac1, Rac2 and β -actin (top left). Western blot of MTLn3 cells treated with control or Rac1 siRNA and blotted for Rac1, Rac3 and β -actin (bottom left), and quantification of Rac3 protein levels in control and Rac1 knockdown conditions (right). $n=3$ independent experiments, error bars are s.e.m. Student's t -test was used. $P=0.3$. Representative blots are shown from 3 blots for each condition. Uncropped images of blots are shown in Supplementary Fig. 9.

the adjacent invadopodium not directly irradiated disappears at 78% frequency under those conditions. Only 8.7% of invadopodia (4/46) remained when an adjacent invadopodium was irradiated and dissolved. As a control, we used a C450A constitutively caged mutant of PA-Rac1 (ref. 27). In this case, photoactivation does not affect the irradiated invadopodium, and approximately 26% of invadopodia dissolved (Fig. 4b–d and Supplementary Video 5).

Pak1–cortactin axis propagates Rac1-dependent invadopodia disassembly

As the evidence pointed to Rac1 inducing invadopodia disassembly, we investigated downstream targets. Two well-studied Rac1 effectors are the Paks and the Wave protein family¹⁸. Previously Wave was shown to have no effect on invadopodia¹³, but Pak was not studied in this context. High levels of Pak1 are correlated with

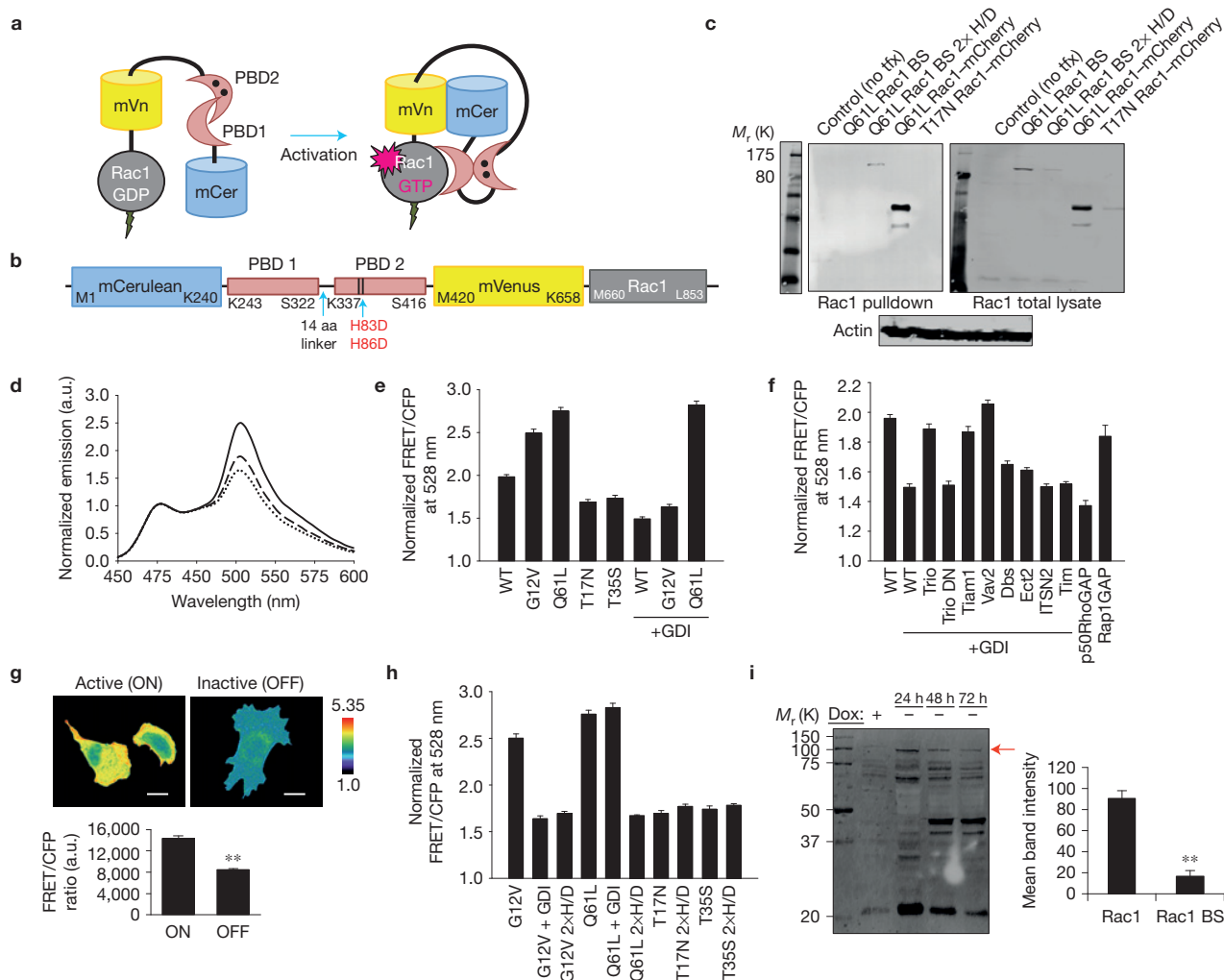


Figure 2 Rac1 biosensor. (a) Biosensor design: inactive (left) versus active (right). Black dots: H83D–H86D point mutations. (b) Biosensor domains. Amino acid residues are indicated. (c) Rac1 biosensor does not bind endogenous effectors. Left: GST–Pak–CRIB pull-down of $M_r \sim 100$ K constitutively active (Q61L) biosensor, Q61L biosensor with point mutations in both binding domains (2×H/D) and controls. Right: total lysates detected for Rac1 and β -actin. Representative image from 3 blots. (d) Characteristic emission spectra of biosensor mutants (excitation: 433 nm, normalized to emission: 475 nm). G12V (active, solid line), T17N (inactive, dashed line) and G12V with 3×-excess GDI (dotted line). Representative fluorometry spectra from 9 wells from 3 independent experiments per condition. (e) Ratios of WT and mutant (G12V, Q61L; T17N; T35S-effector-binding-deficient) versions of biosensor, with or without 3×-excess GDI. $n=9$ wells for each condition, pooled from 3 independent experiments, with s.e.m. P values (Student's t -test): WT versus G12V = 5.49848×10^{-8} ; WT versus Q61L = 8.92796×10^{-11} ; WT versus T17N = 6.39471×10^{-5} ; WT versus T35S = 1.7×10^{-4} ; WT versus WT + GDI = 5.856×10^{-9} ; G12V versus G12V + GDI = 1.59632×10^{-10} ; Q61L versus Q61L + GDI = 0.1353 . (f) Ratios of WT Rac1 biosensor co-expressed with GEF and GAP. Trio, Tiam1 and Vav2 are Rac1 GEFs rescuing biosensor activity under 3×-excess GDI. Trio DN (dominant negative), Dbs, Ect2, ITSN2 and Tim do not have Rac-GEF activity. p50RhoGAP reduces FRET to GDI-inhibited levels. Rap1GAP is shown as control. $n=9$ wells for each condition, pooled from 3 independent experiments, with s.e.m. P values (Student's

t -test): WT biosensor only versus: WT + GDI = 5.85609×10^{-9} ; Trio = 0.07294 ; Trio DN = 8.41187×10^{-9} ; Tiam1 = 0.08062 ; Vav2 = 0.01858 ; Dbs = 8.59088×10^{-7} ; Ect2 = 3.51775×10^{-7} ; ITSN2 = 1.1627×10^{-8} ; Tim = 3.04742×10^{-8} ; p50RhoGAP = 0.009341 ; Rap1GAP = 0.135735 . (g) Overexpression of mutant Rac1 biosensors in mouse embryonic fibroblasts. G12V shows higher ratio (left) than T17N (right). $n=23$ cells for each condition, pooled from 3 independent experiments, with s.e.m. Scale bars, 20 μ m. $**P=7.27558 \times 10^{-8}$ (Student's t -test). Representative image set from 4 image sets. (h) Ratios of G12V and Q61L, T17N and T35S (effector-binding-deficient) mutant versions of the Rac1 biosensor, with and without Rac-binding-deficient mutation in both PBDs (2×H/D). $n=9$ wells for each condition, pooled from 3 independent experiments, with s.e.m. P values (Student's t -test): G12V versus: G12V + GDI = 1.59632×10^{-10} ; G12V 2×H/D = 6.23298×10^{-9} ; Q61L versus: Q61L + GDI = 0.13529 ; Q61L 2×H/D = 1.2788×10^{-9} ; T17N versus T17N 2×H/D = 0.04264 ; T35S versus T35S 2×H/D = 0.136663 . (i) Western blot of MTLn3s with tet-OFF Rac1 biosensor and induced for indicated times and detected for Rac1. Red arrow: biosensor band. Optimal induction = 24 h. Quantification from the 24 h-induction lane, showing protein levels of endogenous Rac1 and Rac1 biosensor (right). $**P=0.00086$ (Student's t -test), $n=3$ independent experiments, with s.e.m. Representative image from 3 blots. Uncropped images of blots are shown in Supplementary Fig. 9.

tumour invasiveness⁴¹. Previous studies showed that inactive Pak1 is constitutively associated with cortactin in a manner that is independent of Rac1 (ref. 42), and when activated by

a GTPase, Pak1 phosphorylates cortactin on Ser 113, causing the release of cortactin from F-actin⁴³ to induce turnover of podosomes⁴⁴. As Pak1 is one of the main downstream effectors of

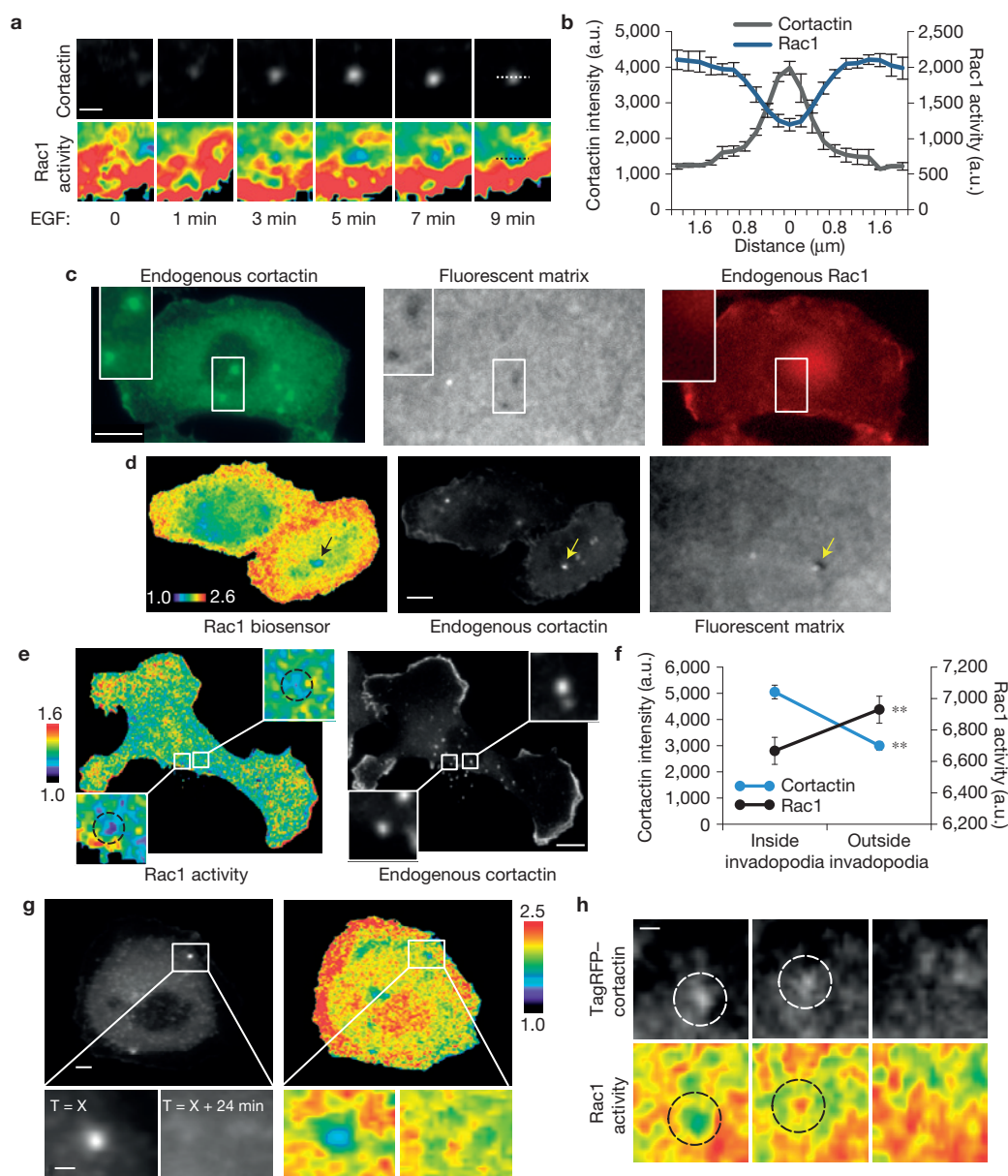


Figure 3 Rac1 activity at invadopodia. **(a)** Close-up of a single invadopodium forming at specific time points after EGF stimulation (Supplementary Video 2). Scale bar, $2\mu\text{m}$. Representative time-lapse panel is shown from 15 cell movies. **(b)** Linescan measurement along invadopodia of Rac1 activity and cortactin fluorescence. Invadopodia measured were approximately $1.3\mu\text{m}$ in diameter, with a small percentage closer to $2\mu\text{m}$. $n=27$ invadopodia, pooled from 6 independent experiments, with s.e.m. $P=0.047$ (Student's *t*-test). The *x* axis is the absolute distance measured. The fluorescence data sets were aligned to the maximal centre value position of cortactin fluorescence taken as the centre of the invadopodia cores. **(c)** Rac1 and cortactin antibodies were used to show endogenous localization of each protein. Unlike cortactin, which is concentrated in actively degrading invadopodia (white outlined insets), endogenous Rac1 (in either GDP- or GTP-bound conformations) is uniformly distributed throughout the cell. Scale bar, $10\mu\text{m}$. Representative image set from 17 image sets. **(d)** Rac1-biosensor-expressing MTLn3 cell plated on Alexa-568-conjugated gelatin and stained for cortactin using Cy5 post-fixation to exclude possibilities of spurious FRET between Venus and TagRFP-cortactin. Arrows point to exclusion of Rac1 activity (left) localized to spots of cortactin accumulation (middle) and holes in the matrix (right), indicating a mature, functional invadopodium. Scale bar, $10\mu\text{m}$. Representative image

set from 22 image sets. **(e)** MDA-MB-231 cell expressing Rac1 biosensor, fixed and stained with antibody against endogenous cortactin. Dashed circles show invadopodia location and exclusion of Rac1 activity. Scale bar, $10\mu\text{m}$. Representative image set from 10 image sets. **(f)** Quantification of cortactin intensity and Rac1 biosensor activity inside and outside cortactin-enriched spots. Rac1 activity is lower inside invadopodia and higher outside, whereas cortactin accumulation is high inside and low outside. $n=19$ invadopodia, pooled from 3 independent experiments, with s.e.m. Student's *t*-test: $**P=1.2486 \times 10^{-10}$ (cortactin), $**P=2.82709 \times 10^{-5}$ (Rac1). **(g)** Representative biosensor-expressing MTLn3 cell at steady state with TagRFP-cortactin marker. Close-up of pre-formed invadopodium ($T=X$), and later time point when the invadopodium disappeared ($T=X+24\text{ min}$). Scale bar, $2\mu\text{m}$. Representative images are shown from 50 cell movies. **(h)** Close-up of another invadopodium in its final stages. White and black circles show the invadopodium location and corresponding Rac1 activity levels. Left: steady-state invadopodia and associated reduced Rac1 activity. Middle: moment of elevated Rac1 activity at the core of invadopodia just before disappearance. Right: the same region after the disappearance of invadopodia. Scale bar, $2\mu\text{m}$. Representative image sets are shown from 4 cell movies that showed elevated activity of Rac1 before invadopodia disappearance. Total steady-state cell movie sets = 50.

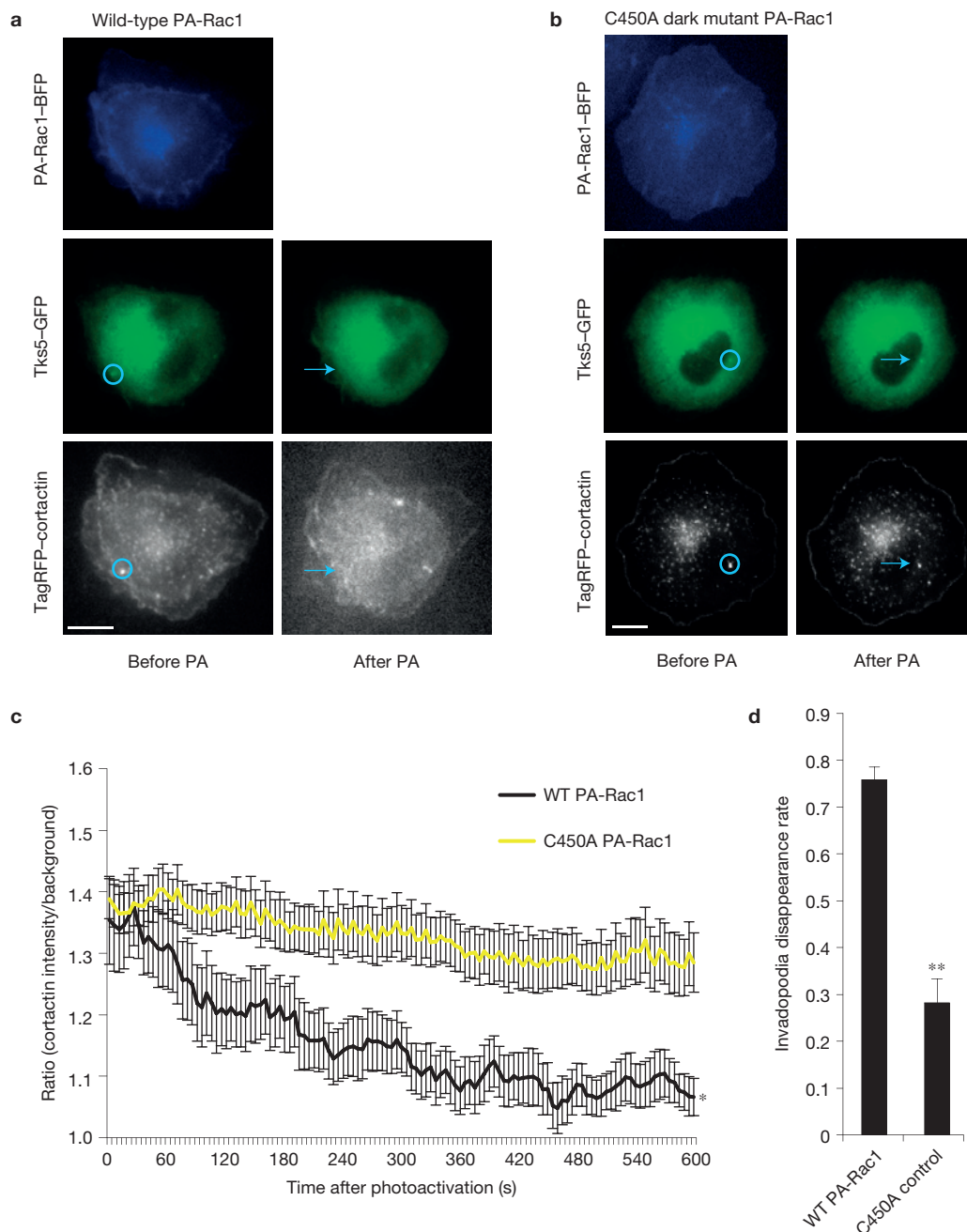


Figure 4 Localized photoactivation of Rac1 induces invadopodia disassembly. **(a,b)** Representative images of MTLn3 cells triple-transfected with either PA-Rac1 **(a)** or C450A PA-Rac1 **(b)**, and two invadopodia markers (Tks5 and cortactin), before and after photoactivation (PA). PA-Rac1 is uniformly distributed throughout the cells and not specifically concentrated at invadopodia structures, similarly to endogenous Rac1 distribution. Blue circles represent photoactivated region. Blue arrows point to same region after photoactivation (from Supplementary Video 4 and 5). Scale bars, 10 μ m. **(a)** Representative image set is shown from 40 image

sets. **(b)** Representative image set is shown from 30 image sets. **(c)** Quantification of **a** and **b**. $n=11$ cells for each condition, pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. $*P=1.19176 \times 10^{-47}$. **(d)** Quantification of percentage of invadopodia that disappear as a result of photoactivation with WT PA-Rac1 and C450A PA-Rac1. $n=46$ invadopodia (PA-Rac1), pooled from 3 independent experiments, $n=19$ invadopodia (C450A), pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. $**P=0.00015175$.

Rac1, we suggested a direct role in linking Rac1 to invadopodia breakdown. Immunofluorescence indicated that endogenous Pak1 co-localizes with cortactin at mature, degrading invadopodia (Fig. 5a), even when Rac1 is depleted (Supplementary Fig. 3a). Next, we used RNAi against Pak1 (Fig. 5b and Supplementary

Fig. 1h), recapitulating the results of Rac1 knockdown by increasing matrix degradation (Fig. 5c and Supplementary Fig. 1a–d). Invadopodia formation was unaffected by Pak1 siRNA, with no change in average invadopodia per cell, but invadopodia lifetime increased (Fig. 5d). Collectively, these results implicated Pak1 as

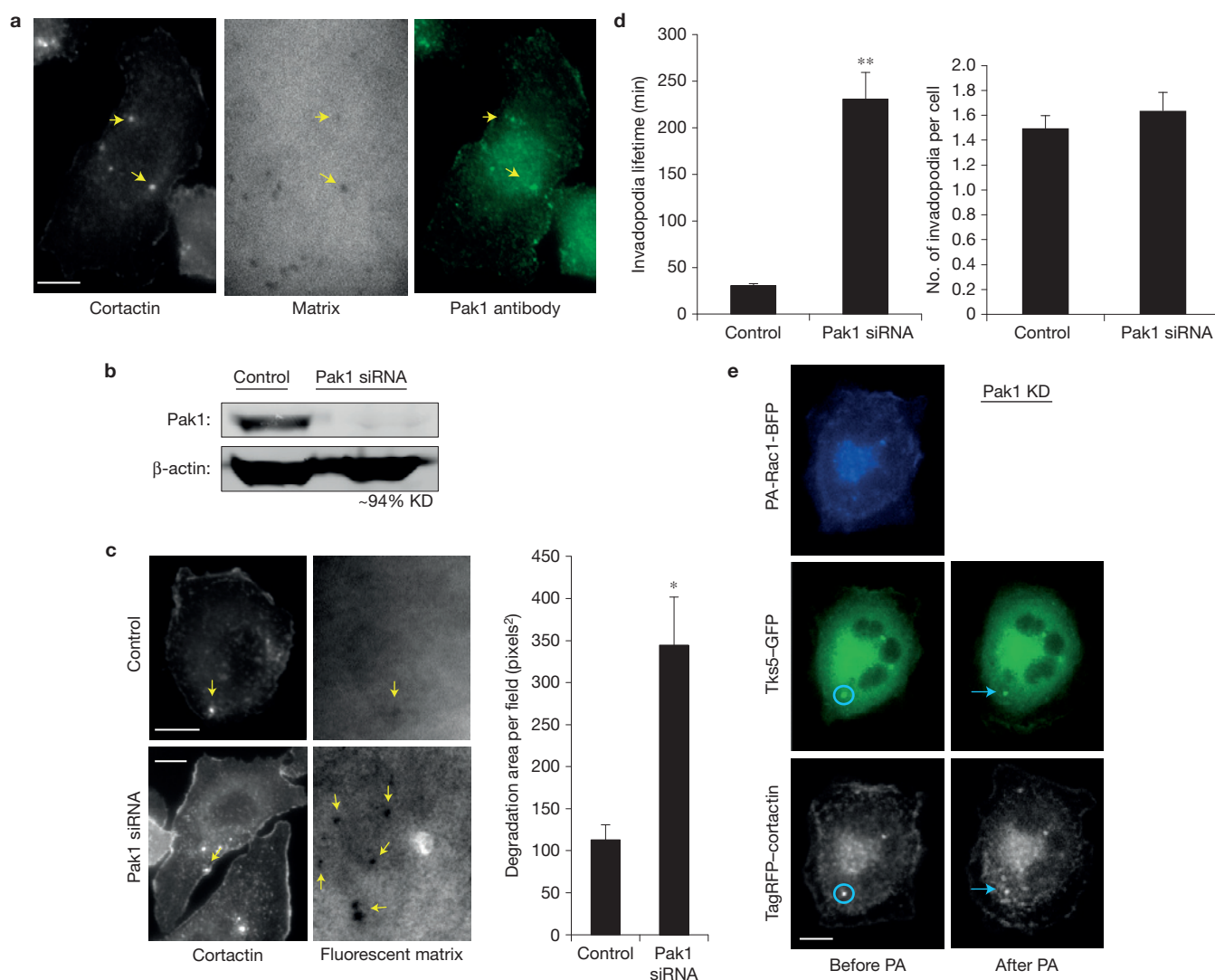


Figure 5 Pak1 links Rac1 to cortactin for invadopodia disassembly. **(a)** MTLn3 cell plated overnight on Alexa-568-conjugated gelatin and fixed and stained with cortactin or Pak1 antibodies to show endogenous localization. Scale bar, 10 μ m. Representative image set is shown from 30 image sets. **(b)** Western blot of cell lysates of control and Pak1-siRNA-treated MTLn3 cells blotted for Pak1 and β -actin. A representative image is shown from 2 blots. **(c)** MTLn3 cells transfected with control siRNA or Pak1 siRNA and plated overnight on Alexa-568-conjugated gelatin, and fixed and stained with cortactin antibody (left). Yellow arrows show mature invadopodia and sites of matrix degradation. Quantification of mean degradation area per field (right). Scale bars, 10 μ m. $n=45$ fields for each condition, pooled from 4 independent experiments, error bars are s.e.m. Student's t -test was used. $*P=0.041$. Representative image set is shown from 40 image sets. **(d)** Quantification of mean invadopodia lifetime in control and

Pak1-siRNA-treated cells, from time-lapse movies over a period of 6 h (left), and average number of invadopodia per cell (right). Invadopodia lifetime was quantified as the time in minutes from the appearance to the disappearance of RFP-cortactin and Tks5-GFP co-localized spots. $n=13$ invadopodia for each condition, pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. $**P=2.883 \times 10^{-12}$, $P=0.364372402$ (number of invadopodia per cell). **(e)** Representative images of a MTLn3 cell transfected with Pak1 siRNA, then triple-transfected with PA-Rac1 and two fluorescent invadopodia markers (cortactin and Tks5) before and after photoactivation (PA) (from Supplementary Video 6). Blue circles represent photoactivated region. Blue arrows point to the same region after photoactivation. Scale bar, 10 μ m. Representative image set is shown from 27 image sets. Uncropped images of blots are shown in Supplementary Fig. 9.

the immediate downstream target of Rac1 at the invadopodia to mediate disassembly.

To determine whether Pak1 is involved in this pathway, we silenced Pak1 and observed invadopodia dynamics on focal uncaging of Rac1. Uncaging PA-Rac1 in the Pak1 knockdown background did not cause the disappearance of invadopodia (approximately 19% invadopodia disappearance), but produced edge ruffling and a lamellipodium (Fig. 5e; Supplementary Fig. 3e and Video 6).

After establishing the Rac1-Pak1 link, we examined whether the phosphorylation of cortactin by Pak1 was important. Focal uncaging of PA-Rac1 with an overexpressed mTagRFP-cortactin containing a non-phosphorylatable point mutation (S113A) where Pak1 is known to phosphorylate cortactin^{43,45} disassembled the invadopodia only 30% of the time, but the leading edge ruffling and lamellipodia protrusions were unaffected (Supplementary Fig. 3b,e and Video 7). Expression of the cortactin mutant alone

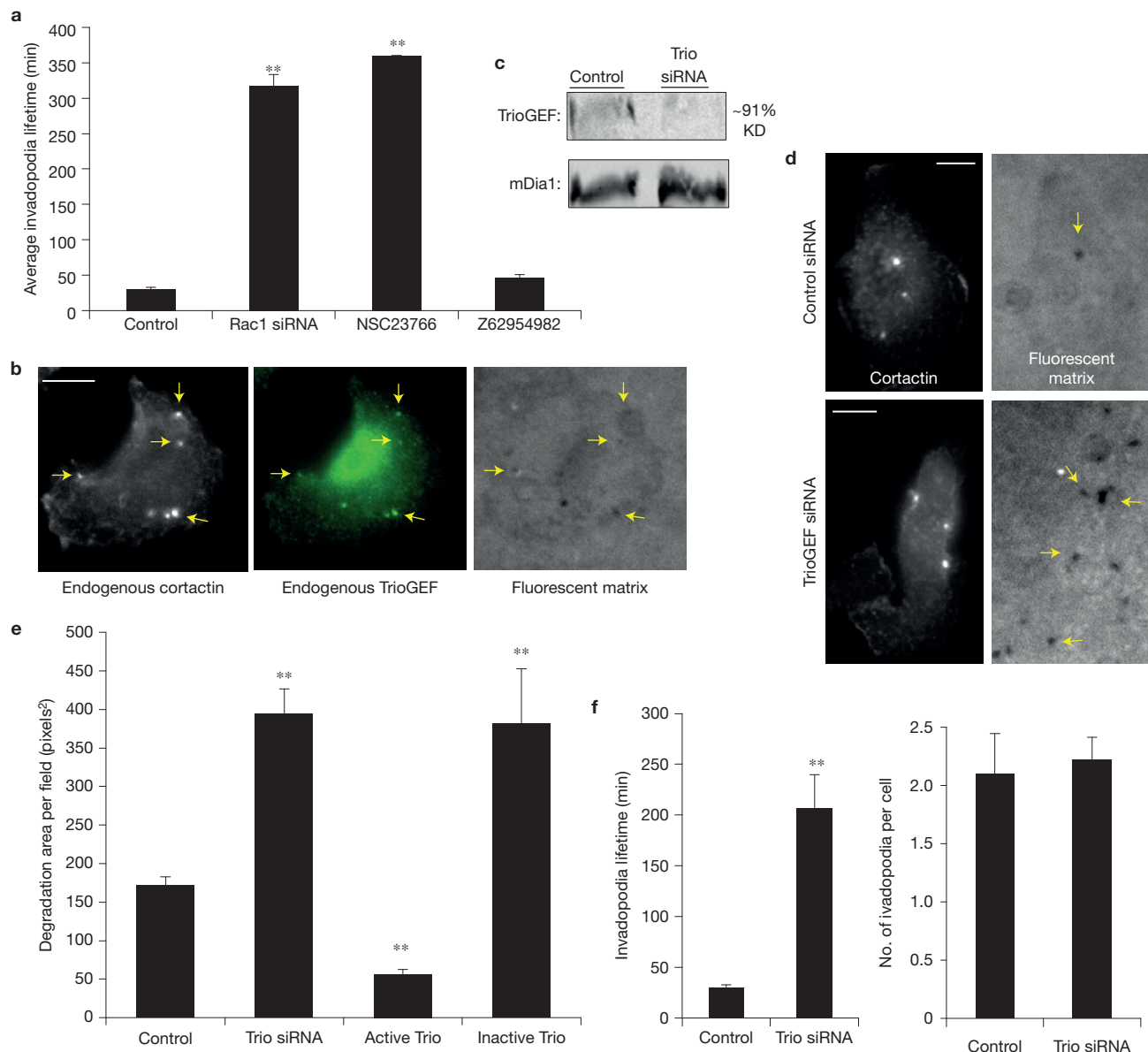


Figure 6 TrioGEF is involved in invadopodia activity. **(a)** Quantification of invadopodia lifetimes after either depleting Rac1 by siRNA or inhibiting Rac1 with drugs. $n=9$ invadopodia for each condition, pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. $**P=6.531 \times 10^{-27}$ (Rac1 siRNA), $**P \leq 2.57 \times 10^{-11}$ (NSC23766). **(b)** Representative images of an MTLn3 cell plated overnight on Alexa-405-conjugated gelatin and fixed and stained with cortactin and TrioGEF antibodies to show endogenous localization. Arrows point to mature invadopodia and sites of matrix degradation. Scale bar, 10 μ m. Representative image set is shown from 41 image sets. **(c)** Western blot of cell lysates of control and Trio-siRNA-treated MTLn3 cells blotted for TrioGEF and mDia1. A representative image is shown from 2 blots. **(d)** MTLn3 cells transfected with control siRNA (top panels) or TrioGEF siRNA (bottom panels) and plated on Alexa-405-conjugated gelatin overnight. Arrows point to sites of invadopodia degradation. Scale

bars, 10 μ m. Representative image set is shown from 68 image sets. **(e)** Quantification of mean degradation area per field after TrioGEF depletion or expression of constitutively active or dominant-negative mutants of Trio. $n=25$ fields for each condition pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. $**P=4.63 \times 10^{-7}$ (Trio siRNA), $**P=5.4 \times 10^{-8}$ (Active Trio), $**P=0.01165$ (Inactive Trio). **(f)** Quantification of mean invadopodia lifetime in control and TrioGEF-siRNA-treated cells, from time-lapse movies over a period of 6 h (left) and average number of invadopodia per cell (right). Invadopodia lifetime was quantified as the time in minutes from the appearance to the disappearance of RFP-cortactin and Tks5-GFP co-localized spots. $n=11$ invadopodia for each condition pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. $**P=6.2151 \times 10^{-10}$, $P=0.380125939$ (number of invadopodia per cell). Uncropped images of blots are shown in Supplementary Fig. 9.

increases matrix degradation similar to depletion of Rac1 or Pak1 (Supplementary Fig. 3c), and increases invadopodia lifetime (Supplementary Fig. 3d).

Moreover, we used the proximity ligation assay (PLA) between cortactin and phospho-serine to measure the total phospho-serine

levels of cortactin at invadopodia demonstrating whether the Rac1–Pak1 pathway leads to changes in the total phospho-serine content of cortactin at invadopodia *in situ*. Total cortactin phospho-serine is reduced significantly when Pak1 was silenced, supporting the link between Pak1 and cortactin (Supplementary Fig. 3f,g).

These results suggest that the Rac1–Pak1 pathway probably produces a destabilization of invadopodia structures through serine phosphorylation of cortactin.

TrioGEF activates Rac1 at invadopodia to induce disassembly

Spatial regulation of GTPases is crucial to their function and because they are activated by GEFs, we sought to determine which GEF may activate Rac1 at invadopodia for disassembly. First, we used the Rac1-inhibitor NSC23766, which inhibits Rac1 activation by TrioGEF and Tiam1 (ref. 46), and performed an invadopodia lifetime study over a 6-h time period. Untreated cells had average invadopodia lifetimes of 30 min (range: 14 min–2 h), measured by mTagRFP–cortactin and Tks5–GFP co-localization (Fig. 6a and Supplementary Video 8). In NSC23766-treated cells, invadopodia did not disappear during the time frame of image acquisition (Fig. 6a and Supplementary Video 9). We used another Rac1-inhibitor Z62954982, which inhibits Rac1–Tiam1 interaction but not TrioGEF (ref. 47). The distribution of invadopodia lifetimes was similar to the control after the Z62954982 treatment, suggesting that Trio may activate Rac1 at invadopodia (Fig. 6a and Supplementary Video 10). Endogenous TrioGEF is localized to invadopodia (Fig. 6b), but endogenous Tiam1 does not (Supplementary Fig. 4a). No Tiam1–GFP accumulated at invadopodia (Supplementary Fig. 4b), and a dominant-negative mutant Tiam1 (ref. 48) did not affect invadopodia functions (Supplementary Fig. 4c). Furthermore, silencing TrioGEF (Fig. 6c and Supplementary Fig. 1h) recapitulates the increase in degradation and invadopodia lifetimes (Fig. 6d,e and Supplementary Fig. 1a–d), but the average number of invadopodia per cell does not change (Fig. 6f), similar to the Rac1 RNAi result. We overexpressed the constitutively activated (Trio-D1SH3; ref. 49) and dominant-negative (N1406A-D1407A) form of the same Trio-D1SH3 (ref. 50), and observed that the active form reduced matrix degradation, but the dominant-negative form produced increased degradation (Fig. 6e). Full-length WT, dominant-negative, and the short dominant-negative Trio-D1SH3 are present in about 20% of invadopodia, but the Trio-D1 Δ SH3-dominant-negative form lacking the tandem SH3 domain cannot localize at invadopodia, and EGF stimulation does not affect the localization of full-length Trio (Supplementary Fig. 5a–c). This means that Trio is recruited transiently to invadopodia through its SH3 domain at DH-PH-1 (Supplementary Fig. 5d) because less than 30% of invadopodia have Trio accumulation. Moreover, Trio is increased at invadopodia when endogenous Rac1 is silenced (Supplementary Fig. 5e), probably as a consequence of its disassembly defect. Trio knockdown lowers the global Rac1 activity levels in cells (Fig. 7a,b), making the difference between the inside and outside of invadopodia less pronounced (Fig. 7c). Furthermore, we performed PLA under Trio knockdown and found that the total phospho-serine levels on cortactin at invadopodia decreased significantly (Supplementary Fig. 3f,g).

An invadopodia-associated complex of cortactin, paxillin and PKC has been shown previously⁵¹, and the Git1–Pix–Pak complex can be recruited to paxillin through Git1 (ref. 52). Pix proteins are GEF for Rac1 and Cdc42 (ref. 53). α - and β -Pix both localize to invadopodia and this recruitment is unaffected by EGF stimulation; however, knocking down either of the endogenous pix isoforms reduced the amount of matrix degradation (Supplementary Fig. 6a–e). As this is the opposite phenotype to Rac1, Pak1 or TrioGEF knockdowns, the Pix proteins are

probably involved during other aspects of invadopodia dynamics, but not in disassembly. Moreover, paxillin does not accumulate in the core of the invadopodia, suggesting that Pix localization at invadopodia is not paxillin-mediated (Supplementary Fig. 6f).

Trio–Rac1–Pak1 axis impacts invasion in three-dimensional environment

The pro-invasive role of Rac1 in aggressive tumours is documented in a number of systems^{15–20}. We sought to determine whether the Trio–Rac1–Pak1 axis impacts three-dimensional (3D) invasion. Invasion is significantly impacted when Trio, Rac1 or Pak1 is silenced in metastatic tumour cells but not in MCF10A cells (Fig. 8a and Supplementary Fig. 1a–f). Motility in 3D collagen matrices is significantly impaired on Rac1, Pak1 or Trio depletion (Fig. 8b–d). Furthermore, matrix degradation in 3D is increased in the absence of the Trio–Rac1–Pak1 axis (Fig. 8e,f), suggesting that the same mechanism that aberrantly enhances the matrix degradation in 2D also operates under 3D conditions. These results suggest that proper coordination of matrix degradation and locomotory protrusions is required for efficient invasion in 3D environment.

DISCUSSION

We report the localized activation dynamics of Rac1 directly regulating the stability and disassembly of invadopodia through Pak1 and cortactin phosphorylation in metastatic breast carcinomas (Fig. 8g). We show that TrioGEF activates Rac1 at invadopodia, and this Trio–Rac1–Pak1 cascade is regulated independently from the lamellipodia compartment. This is probably through different immediate downstream effectors, because depleting either Trio or Pak1 did not abrogate EGF-stimulated lamellipodia protrusion, but depleting Rac1 did (Supplementary Fig. 7a). Importantly, we provide conserved observations for the Trio–Rac1–Pak1 axis in metastatic breast carcinomas but not in normal epithelial cells; a critical distinction in the signalling mechanisms used to affect efficient invasions between malignant versus normal cells.

As invadopodia are transient subcellular structures, we approached the problem by building a biosensor for Rac1. The fundamental difference between our sensor and the previous-generation system³⁴ is that the latter is constitutively anchored to the plasma membrane through the k-Ras CAAX box^{34,35}, producing GEF–GAP sensors. In our system the sequestration by GDI is correctly taken into account³³.

Our data and other studies suggested that invasive tumours use two separate downstream signalling pathways to achieve efficient migration on Rac1 activation. The lamellipodia driven by Rac1 is Wave2-dependent^{13,54}; indeed, we show here that in a Pak1-silenced background, photoactivation of Rac1 at an invadopodium does not result in its disassembly, and edge ruffling and lamellipodia protrusions are unaffected. The mechanism by which Pak1 enhances the turnover of invasive processes agrees with other studies suggesting Pak1 inducing the turnover of podosomes^{43,44}, and cortactin phosphorylation by Pak destabilizing F-actin–cortactin association⁴³. These studies and our results support the idea that the Rac1–Pak1 axis induces the turnover of many of these adhesive and invasive processes, including focal adhesions^{55–57}.

Invadopodia formation is a multistep process⁸. Efficient turnover of invadopodia seems critical to effective invasion. Previously, focal

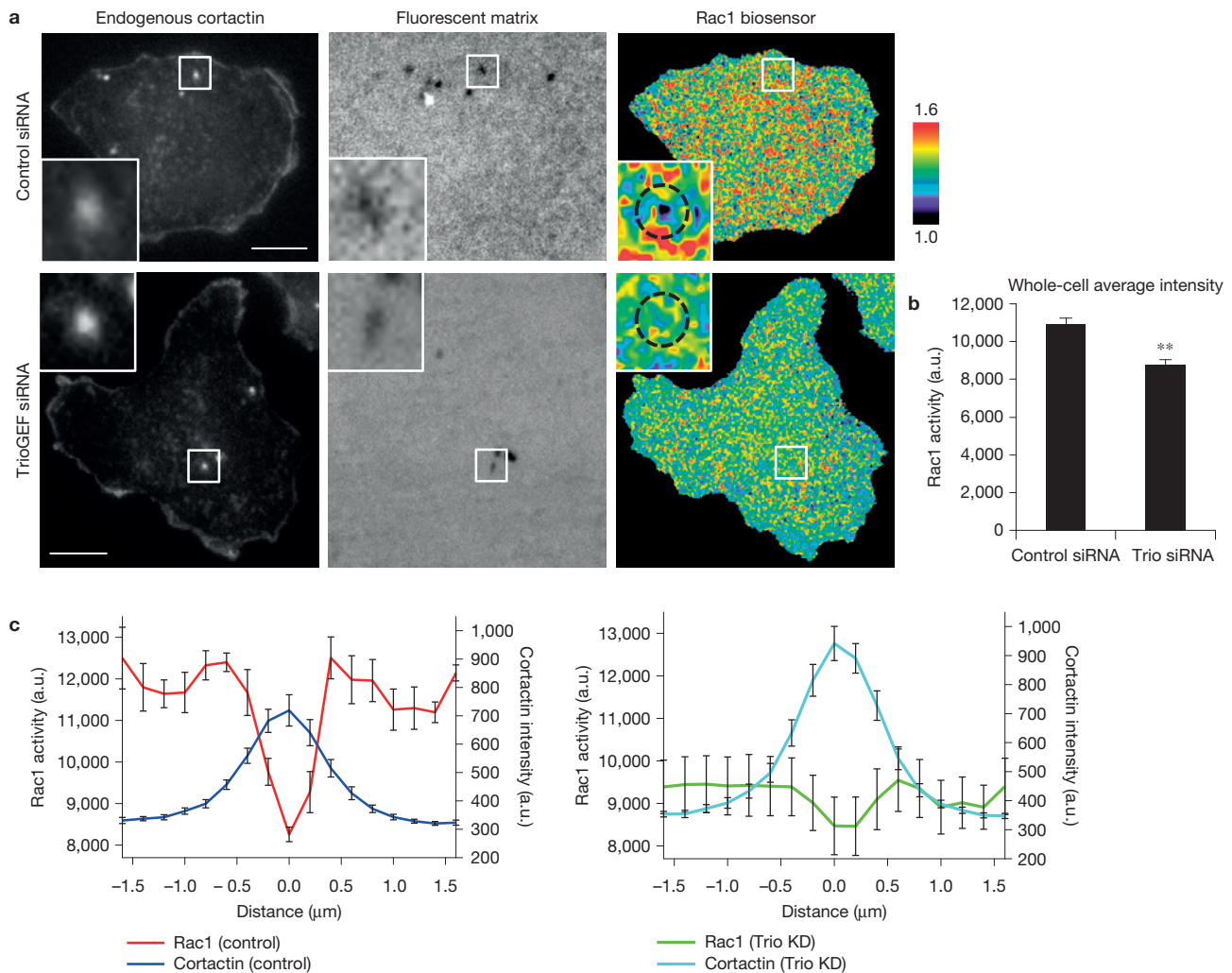


Figure 7 TrioGEF affects Rac1 biosensor activation levels. **(a)** A MTLn3 cell expressing Rac1 biosensor and treated with control or Trio GEF siRNA plated on Alexa-405-conjugated gelatin and fixed and stained for endogenous cortactin. White squares are magnified (bottom panels) to show a mature, degrading invadopodium. Black circle highlights Rac1 activity corresponding to the invadopodium. Scale bars, 10 μm . Representative image set from 70 image sets. **(b)** Quantification of average whole-cell Rac1 biosensor activity in control and TrioGEF RNAi-depleted cells. Knocking down Trio causes Rac1

activity levels to drop outside the invadopodia. $n=18$ cells for each condition, pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. $**P=1.50643 \times 10^{-5}$. **(c)** Quantification of cortactin intensity in relation to Rac1 biosensor activity at invadopodia in control and TrioGEF-siRNA-treated cells. $n=14$ invadopodia for each condition, pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. $P=3.1981 \times 10^{-10}$, comparing the average Rac1 activity levels outside the invadopodia between the control versus the TrioGEF knockdown.

adhesion kinase was reported to antagonize c-Src to regulate the total number of invadopodia in MTLn3 cells; thus, overexpression of c-Src can produce greater numbers of structures⁵⁸. We show that the number of structures is independently regulated by c-Src (refs 8,58,59) and not the Trio–Rac1–Pak1 axis (Supplementary Fig. 7b,c).

Cancer invasion *in vivo* is a complex process involving localized regulations of signalling proteins, including GTPases. GEFs are established spatial activators of GTPases (ref. 37). The specific mechanism of TrioGEF control is not well known; however, we established a role for Trio as the activator of Rac1 at invadopodia. Trio contains two DH–PH domains, which target RhoG and Rac1, or RhoA (ref. 60). In glioblastoma, RhoG is involved in invadopodia formation²⁵. In breast carcinoma, siRNA of RhoG has no effect on matrix degradation nor does RhoG accumulate at invadopodia (Supplementary Fig. 8). The difference could be because glioblastoma

is not derived from epithelium and invades brain tissue locally; thus, the ECM environment is much different in brain than epithelium⁶¹. We showed that the SH3 domain of Trio is required for transient invadopodia localization (Supplementary Fig. 5d). These observations clearly point to the importance of localized regulation of GTPases within subcellular compartments; indeed, when TrioGEF was silenced, overall Rac1 activity was reduced by approximately 20% (Fig. 7). Although this had no effect on EGF-stimulated leading edge protrusion (Supplementary Fig. 7a), it clearly impacted invadopodia as both the lifetimes and matrix degradation were significantly altered. Thus, the functional regulation of how Trio activates Rac1 only at a specific time and in a context of invadopodia disassembly needs to be further explored. Also, other mechanisms may cooperate with the Trio–Rac1–Pak1 axis during turnover of invadopodia⁵⁹.

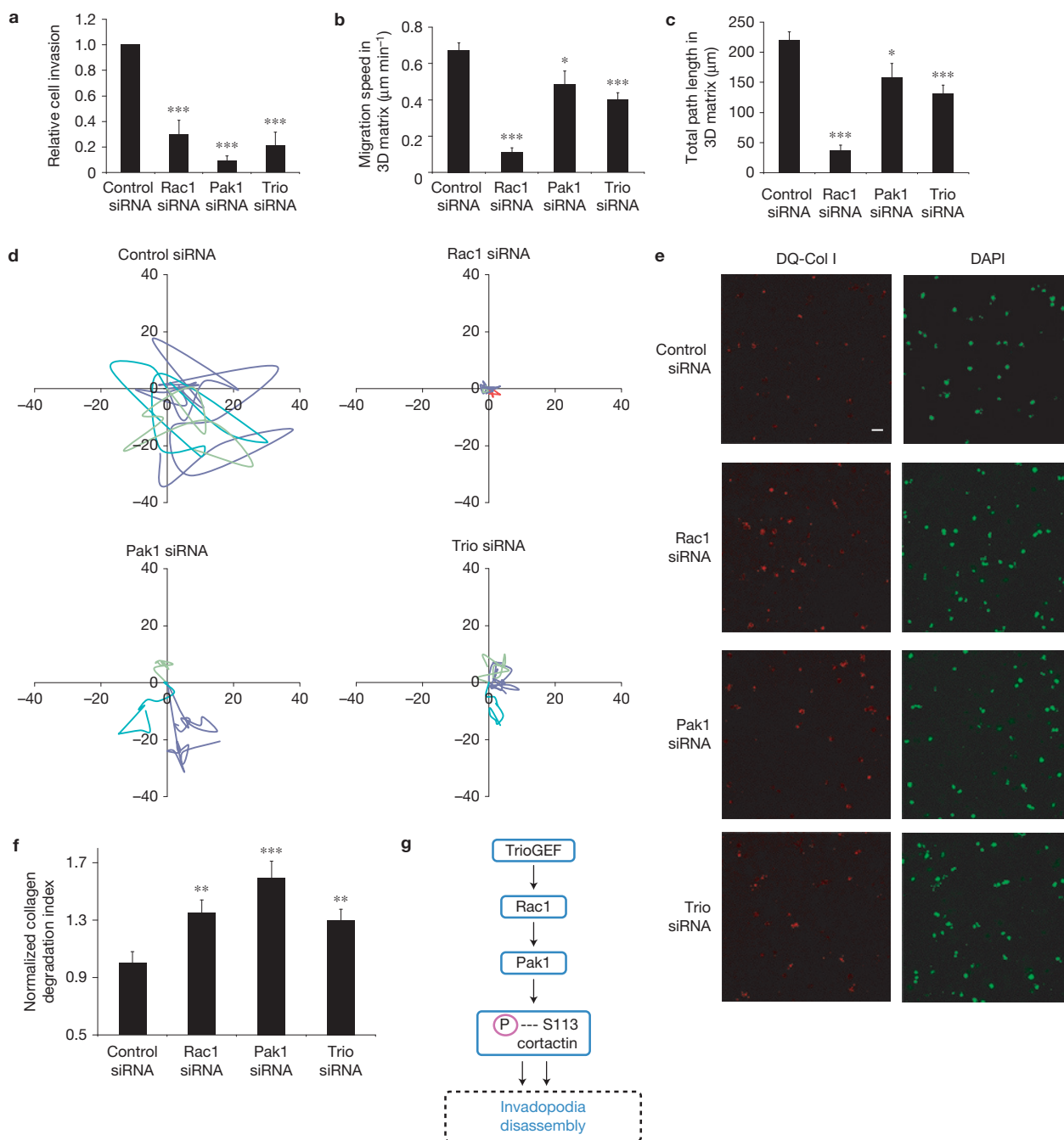


Figure 8 Trio–Rac1–Pak1 signalling axis is necessary for efficient invasion and 3D motility. **(a)** MTLn3 cells transfected with control, Rac1, Pak1 and Trio siRNA show reduced invasion through Matrigel. $n=12$ fields pooled from 3 independent experiments per condition, error bars are s.e.m. Student's t -test was used. *** $P=0.00065$ (Rac1), *** $P=5.2 \times 10^{-7}$ (Pak1), *** $P=0.00039$ (Trio). **(b)** MTLn3 cells transfected with control, Rac1, Pak1 and Trio siRNA show reduced migration speed in 3D matrices. $n=24$ cells from one experiment per condition were analysed, error bars are s.e.m. Student's t -test was used. *** $P=1.88 \times 10^{-15}$ (Rac1), *** $P=1.32 \times 10^{-5}$ (Trio), * $P=0.022$ (Pak1). **(c)** MTLn3 cells transfected with control, Rac1, Pak1 and Trio siRNA show reduced total distance migrated by cells over time in 3D matrices. $n=24$ cells from one experiment per condition were analysed, error bars are s.e.m. Student's t -test was used. *** $P=7.86 \times 10^{-16}$ (Rac1), *** $P=2.24 \times 10^{-5}$ (Trio), * $P=0.023$ (Pak1). **(d)** Representative plots of x–y displacement (in micrometres) of 4 individual cell traces of cells moving in 3D matrices. Each panel shows 4 representative cell motility

traces, out of a total of 24 cell traces per condition. **(e)** Representative images of the maximum projections of 100 μm stacks of MTLn3 cells treated with Trio, Rac1 or Pak1 siRNA and embedded in a 3D collagen-Matrigel matrix containing DQ-collagen. DQ-Col I intensity and DAPI staining is shown. Scale bar, 50 μm . Representative image set from 17 image sets for the control condition, from 18 image sets for Rac1 siRNA, from 17 image sets for Pak1 siRNA, and from 19 image sets for Trio siRNA. **(f)** Quantification of mean DQ-collagen (degradation) per field in MTLn3 cells treated with control, Rac1 Pak1 or Trio siRNA. $n=16$ fields per condition, pooled from 3 independent experiments, error bars are s.e.m. Student's t -test was used. ** $P=0.0059$ (Rac1), *** $P=0.00017$ (Pak1), ** $P=0.009$ (Trio). **(g)** Model for the regulation of invadopodia turnover. For invadopodia to disassemble, Rac1 needs to be activated by TrioGEF. Downstream of Rac1, Pak1 phosphorylates cortactin at Ser 113, releasing cortactin and presumably mediating the destabilization of the F-actin filaments, disassembling the local actin cytoskeleton and ultimately the invadopodium structure.

On the basis of our observations, we propose two models where the Rac1-mediated disassembly of invasive protrusions could be advantageous for tumour cells. In 3D invasion *in vivo*, invadopodia and lamellipodia compartments converge into a single leading front⁶². Rac1 activation at the leading front propagates through two separate downstream effector pathways to mediate: disassembly and clearance of components necessary for matrix degradation (through Pak1); and simultaneously, extend protrusions with locomotive characteristics as opposed to degradative (through Wave2). This is an efficient mechanism for switching between these two characteristics by a single upstream regulator. This model is consistent with reports of Rac1 producing pro-invasive phenotypes²⁰, because an ability to increase the invadopodia turnover could enhance the ability of tumours to switch rapidly between invasive versus locomotive protrusions. Indeed, cells depleted of Trio–Rac1–Pak1 have motility defects in 3D matrices (Fig. 8a–d). Alternatively, invasive cells *in vivo* continuously probe the microenvironment within the ECM to detect regions optimal for invasion⁶³. Thus, microenvironmental cues could provide feedback to drive the turnover of degradative protrusions through regulation of localized Rac1 activity modulating the local turnover rates of lamellipodia versus invadopodia. In both models, our observations are consistent with the idea that the turnover of invadopodia is directly regulated through a mechanism activated by Rac1. Under normal conditions, invasive and locomotory protrusions are both cyclical and coordinated, where the end of one is coupled to the start of the other. Thus, when one component is perturbed, the entire cell motility cycle and the invasion machinery become stalled, resulting in aberrantly increased local matrix degradation (Fig. 8e,f) but impaired overall invasion and motility in 3D (Fig. 8a–d). This coordination is critical to efficient invasion and migration in 3D where all pieces of the system need to be functional, unlike in stimulated protrusions in 2D where the initial phase of protrusion formation can be unaffected by Pak1 or Trio depletion but not Rac1 (Supplementary Fig. 7a). By enhancing the turnover dynamics of invadopodia, Rac1 contributes to efficient cycling of matrix degradation versus locomotory protrusions in invasive tumours, allowing for facile cell migration in 3D. □

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

Y.M. and L.H. designed and built the biosensor. Y.M., J.J.B.-C. and L.H. designed the biological experiments. Y.M., J.J.B.-C. and V.M. performed the experiments. J.C. and L.H. advised and gave critical feedback. L.H. provided financial support. J.C. provided reagents and expertise on specific experimental protocols. Y.M. wrote the

original manuscript. J.J.B.-C. and L.H. revised the manuscript. J.J.B.-C., V.M. and L.H. finalized the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Cell lines. MTLn3 were cultured and transfected as previously described¹⁰.

MDA-MB-231 (ATCC HTB-26) and BT-549 (ATCC HTB-122) were cultured in DMEM with 10% fetal bovine serum (FBS), antibiotics (Invitrogen) and insulin (for BT-549) (Invitrogen) as suggested by ATCC.

MCF10A (ATCC CRL 10317) were cultured following the ATCC protocols. MCF10A were transfected as previously described²³. MDA-MB-231 and BT-549 were transfected by nucleofection according to manufacturer's protocols (Lonza). All cell lines were mycoplasma tested.

Intramolecular biosensor for Rac1. The biosensor for Rac1 was subcloned within the multiple cloning site (MCS) of pTriEX-His-Myc4 (Novagen) between the NcoI and XhoI restriction sites. The construct consisted of the monomeric Cerulean (mCerulean) fluorescent protein⁶⁶, a BamHI restriction site, two tandem p21-binding domains (PBD; amino-acid residues 70–149) from Pak1, separated by a HindIII restriction site and GSGGPPGSGSG linker, the monomeric Venus (mVenus) fluorescent protein⁶⁷, an EcoRI restriction site, a full-length Rac1, and a XhoI site immediately following the stop codon. mCerulean was subcloned between the NcoI and BamHI MCS restriction sites using the primer pair 5'-CCATGGTGAGCAAGGGCGAGGAG-3' and 5'-GGATCCTTTGTACAGCTC GTCCATG-3'. The first PBD was subcloned between the BamHI and HindIII sites using the primer pair 5'-GGATCCAAAGAGCGGCCAGAGATTCTCT-3' and 5'-AAGCTTTCCGCCAGACCCTGACTTATCTGTAAAGCTCATGTAT-3'. The second PBD with H83D and H86D GTPase-binding-deficient point mutations was subcloned between the HindIII and NotI sites using the primer pair 5'-AAGCTTCCACAGGGTCTGGAGGCTCCGGGAAAGAGCGGCCAGAGATTCTCTCC-3' and 5'-GCTTCCGCTGACTTATCTGTAAAGCTCATG-3'. Point mutagenesis was performed using the Quikchange PCR mutagenesis kit (Stratagene).

mVenus was subcloned between the NotI and EcoRI sites using the primer pair 5'-GCGGCCCAATGGTGAGCAAGGGCGAGGAGCT-3' and 5'-CTTGTACAG CTCGTCCATGCCGAATTC-3'. Full-length Rac1 was subcloned between the EcoRI and XhoI sites using the primer pair 5'-GAATTCATGCAGGCCATCAAGTGTTGG-3' and 5'-TTACAACAGCAGGCATTTCTCTCGAG-3'.

For production of the tetracycline-inducible retroviral construct, the entire biosensor cassette was cut out of the pTri-EX backbone using NcoI and XhoI restriction sites, blunt-ended, and ligated into the multiple cloning site of the pRetroX-Tight-Pur vector (Clontech) at the NotI site that was blunt-ended.

The design of the biosensor is such that the built-in binding domain does not compete against the binding of endogenous targets by the activated biosensor (Fig. 2c). This feature is critical to reducing potential dominant-negative or overexpression artefacts.

Biosensor validation—fluorometry assay. The biosensor expression construct was transfected into LinXE cells plated 8×10^5 overnight over poly-L-lysine in 6-well plates, using Lipofectamine Plus (Invitrogen) according to the manufacturer's protocols. The total amount of DNA was 700 ng per well. In cases where the biosensor was co-transfected with upstream regulators, the biosensor/GDI ratio was 1:3, and the biosensor/GDI/GEF ratio was 1:3:1–2. At 48 h after transfection, cells were fixed with 3.7% formaldehyde in PBS and their fluorescence spectrum was measured using a MicroMax plate reader attached to a Fluorolog MF2 spectrofluorometer (Horiba Jobin Yvonne). Cells were excited at 433 nm and a fluorescence emission spectrum was measured between 450 and 600 nm. Background autofluorescence was subtracted from the raw spectra, and normalized to CFP emission at 475 nm as previously described³³.

Invadopodia degradation assay. All imaging experiments were performed as previously described^{10,68}. Alexa-568 or -405-conjugated thin gelatin matrix (Invitrogen) was prepared on MatTek dishes (MatTek) as previously described⁶⁹. Gelatin degradation was measured by quantifying the average area of non-fluorescent pixels per field. Approximately 15 random fields were imaged per condition and each independent experiment was performed at least 3 times and averaged. Invadopodia were identified by cortactin and Tks5 staining and manually counted from images.

Reagents and antibodies. mTagRFP-cortactin has been previously described⁸. Cortactin antibody was purchased from Abcam (ab33333 clone 0.T.21; used at 1:200). Rac1 (sc-95 clone C-11), Pak1 (sc-882 clone N-20), TrioGEF (sc-28564 clone H-120), Tiam1 (sc-872 clone C-16), β -actin (sc-69879 clone AC-15), β -Pix (sc-136035 clone 23) and α -Pix (sc-10927 clone Q-20) antibodies were purchased from Santa Cruz Biotechnologies. RhoG (04-486 clone 1F3 B3 E5), pSerine (AB1603 polyclonal), Rac2 (07-604 polyclonal) and Rac3 (07-2151 polyclonal) antibodies were purchased from Millipore. mDia1 (610848 clone 51-mDia1) and paxillin (610051 clone 349-Paxillin) antibodies were from BD Transduction Laboratories.

All primary antibodies were used at 1:200 dilution. siRNA smart pools for Pak1 (L-091307-02), Trio (L-087912-01), RhoG (L-083717-02), β -Pix (L-098943-02) and α -Pix (L-098102-02) and single oligonucleotides for Rac1 (LQ-080171-02), Pak1 (LU-091307-02) and Trio (LU-087912-02) were purchased from Dharmacon (Thermo Scientific) or Qiagen (Rac1: 5'-AAAGAGAUCGGUGUCUGCAAA-3'; ref. 28) and transfected with Oligofectamine (Invitrogen). Human siRNA smart pools for Rac1 (L-003560), Trio (L-005047) and Pak1 (L-003521) were purchased from Dharmacon.

NSC23766 was purchased from EMD Biosciences and dissolved in water. Cells were treated with 100 μ M of the inhibitor before imaging ($IC_{50} = 50 \mu$ M; refs 46,70). Rac1 inhibitor II (Z62954982) was purchased from EMD Millipore and dissolved in dimethylsulphoxide. Cells were treated with 25 μ M of this compound 1 h before imaging ($IC_{50} = 12.2 \mu$ M; ref. 47).

Tiam-1 PH-CC-Ex (amino acids 431–670) competitive inhibitor⁴⁸ was PCR amplified from the mouse full-length GFP-Tiam-1 by using the primer set 5'-GC CATATATTGGCCATATTCTCGAGCTCAGGGCACTGTGCGGAAGGCTGGA G-3' and 5'-GCAATTATATTGACCTAAAGAATTCTTAAGTCCGGGCTGCCAC CAGGGCATGA-3' and ligated into XhoI-EcoRI sites within the multiple cloning site of pEGFP-C1 backbone (Clontech). The Tiam-1 catalytically dead dominant-negative mutant (L1200R-L1201S) version was produced by site-directed mutagenesis using the primer pair 5'-GCGCAACAGCTCCCTGCTCCGAA GCGGGTACTTGAGG-3' and 5'-CCTCAAGTACCGCTTCGGAGCAGGGGAGC GTTTTGGCG-3'.

The TrioGEF dominant-negative catalytically dead (N1406A-D1407A) version was produced by site-directed mutagenesis using the primer pair 5'-CTG AGGTGCATGGCAGCGCGCTCGCTTCGCGAC-3' and 5'-GTGCCGAGCG AGCCGCTGCTGCCATGCACCTCAG-3'.

Full-length α -Pix-GFP was a kind gift from G. Rosenberger⁶⁴.

Full-length wild-type c-Src-GFP, constitutively activated Y528F c-Src-GFP and the dominant-negative K296R-Y528F c-Src-GFP were kind gifts from M. Way⁶⁵.

Western blotting. Whole-cell lysates were prepared by collecting cells and resuspending them in cold lysis buffer (RIPA, plus 4 mM phenylmethyl sulphonyl fluoride and protease inhibitor cocktail), incubating them on ice for 30 min, and then spinning them for 30 min and collecting the supernatant. Blots were analysed using the Odyssey Infrared Imaging System (LI-COR). For quantification, the average grey values of bands were measured with ImageJ software.

Stable cell lines. The Tet-inducible Rac1 biosensor-expressing MTLn3 cell line was generated by using GP2-293 packaging cells to produce retrovirus for infection. MTLn3 parental cells were stably transduced under G-418 selection with the Retro-X Tet-Off Advanced retroviral vector system (Clontech) as previously described¹⁰. The Tet-Off MTLn3 cells were transduced again using the retrovirus produced from GP2-293 cells transfected with the pRetroX-Tight-Pur viral vector containing the biosensor within its MCS, and the stable transductants were selected using puromycin at 10μ g ml⁻¹ as previously described³³, in the presence of 2μ g ml⁻¹ doxycycline to repress the biosensor expression. The cells were then FACS sorted to obtain a population of uniform but low biosensor expression levels. For imaging experiments, cells were induced by removing the doxycycline as previously described³³. Appropriate biosensor expression levels ($\leq 20\%$ of endogenous Rac1) were achieved at 24 h following the induction. For live-cell imaging, additional constructs, when required, were transiently transfected into induced cells 16 h before the start of the assays using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocols.

Live-cell imaging. MTLn3 cells stably expressing the Rac1 biosensor were induced and transfected with mTagRFP-cortactin⁸ and plated on thin-gelatin-matrix-coated glass coverslips 16 h before imaging. For EGF-stimulation experiments, cells were starved for 3 h before image acquisition in L15 medium (Invitrogen) supplemented with 0.35% BSA, then treated with 5 nM EGF for stimulation. Imaging was performed in L15 medium with 5% FBS and Oxyfluor reagent (Oxyrase), in a closed chamber for steady-state experiments.

Rac1 activation levels were measured in living cells by monitoring the ratio of FRET to mCerulean emissions, following previously described methods³³, then mTagRFP-cortactin images were acquired at each time point. Time-lapse sequences were acquired on a custom Olympus IX81ZDC inverted microscope with a beam splitter that enabled simultaneous acquisitions of both FRET and CFP channels using two Coolsnap ESII CCD (charge-coupled device) cameras (Roper Photometrics) mounted on the left side 100% throughput port through an Olympus beam-splitter module, and a set of excitation-emission filter wheels to direct the mTagRFP emission to a third Coolsnap HQII camera (Roper Photometrics) mounted on the bottom 100% throughput port of the microscope. Images were obtained using an Olympus $\times 60$ PlanApoN 1.45 NA UIS2 DIC lens and Metamorph software. Filter sets used for the ratiometric imaging were: mCerulean: ET436-20X, ET480-40M

(Chroma Technology); FRET: ET436-20X, ET535-30M (Chroma Technology); mTagRFP: FF585-29, FF628-32 (Semrock).

The primary fluorescence dichroic turret used a 10/90 reflection/transmittance mirror (Olympus) that provided the compatibility for all of the band-pass filter sets used. Cells were illuminated with a 100 W Hg lamp through a 10% transmittance neutral density filter. At each time point, mCerulean and FRET images were recorded simultaneously for 700 ms with binning 2×2 . An mTagRFP–cortactin image was then acquired with 500 ms exposure at 2×2 binning.

Invadopodia lifetime was measured in MTLn3 cells expressing TagRFP–cortactin and Tks5–GFP, plus various experimental conditions. Cells were plated on thin gelatin matrix and imaged for 6 h with 2 min between frames. Lifetime was quantified by measuring the time from appearance of cortactin and Tks5 colocalized spots to time of disappearance.

Image processing. Metamorph software (Molecular Devices) was used to perform image analysis, as previously described³³. Briefly, images were dark-current, shading-corrected and background-subtracted, followed by a nonlinear coordinate transformation to achieve a pixel-by-pixel matching of all three camera channels. Binary masks generated through intensity thresholding were applied to each emission channels, and the matched FRET and mCerulean image sets were then ratioed to depict Rac1 activation throughout the cell. A linear pseudocolour lookup table was applied, and the ratio values were normalized to the lower scale value. The ratio was corrected for photobleaching using a previously described approach³³.

Photoactivation. MTLn3 cells were triple-transfected with photoactivatable Rac (PA-Rac; ref. 27) fused to mTagBFP, mTagRFP–cortactin, and Tks5–GFP using Lipofectamine 2000, one day before imaging, and plated on thin-gelatin-coated glass coverslips overnight. Spots (3 nm) where invadopodia were present were irradiated with 1 s pulses of 457 nm light, every 30 s. Cortactin and DIC images were acquired every 5 s for 10 min. Metamorph software was used to measure RFP and GFP intensities.

Invasion assay. A Transwell invasion assay (number 354483 BD, Bioscience) was performed and analysed as previously described¹⁰.

Three-dimensional motility and matrix degradation assay. Preparation of 3D cultures was done as previously described⁶⁸.

3D motility experiments were performed using a Delta Vision microscope under a $\times 20$ magnification objective lens and imaged every 5 min for 6 h.

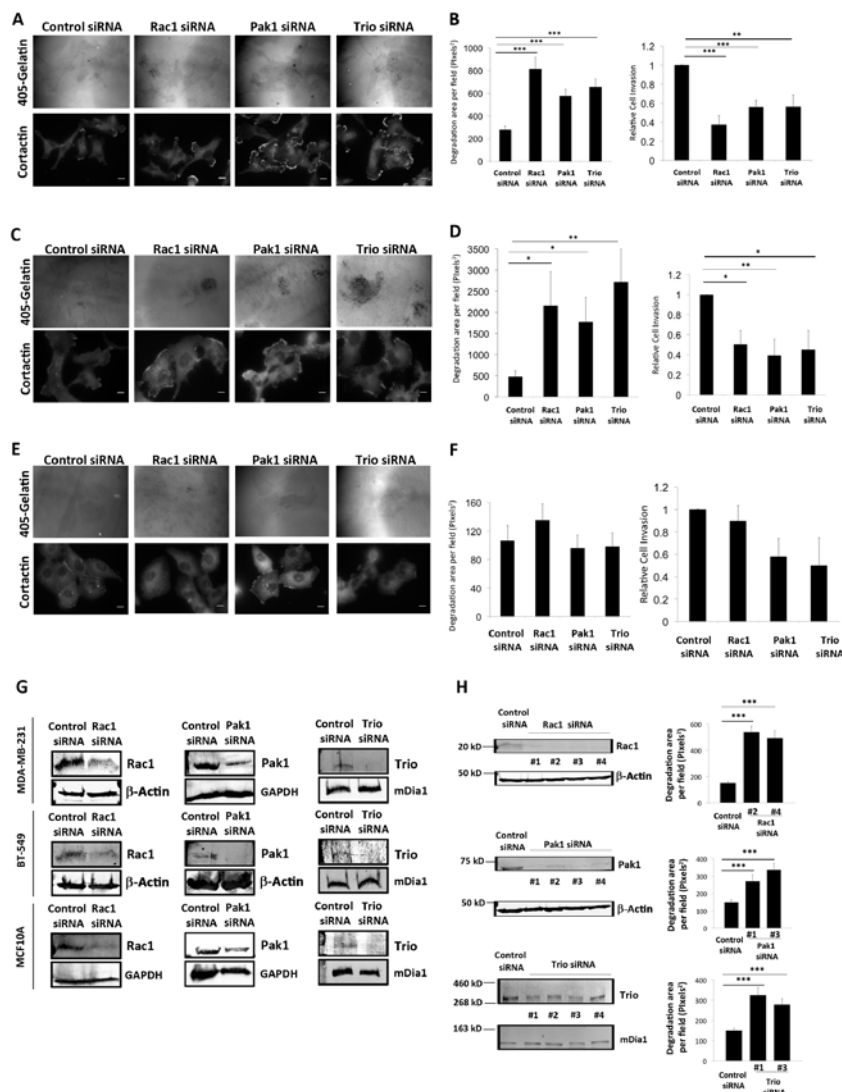
For DQ-collagen degradation experiments, imaging was performed using a Leica SP5 confocal microscope with a $\times 20$ magnification objective lens. Cells were cultured in a mixture of collagen–Matrigel–DQ-collagen 3D matrices for 16 h before fixation and staining with DAPI. Z-stacks of 100 μm depth at 5 μm Z-increments were acquired and the DQ-collagen degradation index was calculated as summation of the mean fluorescence intensity per Z-slice over the total number of planes per field in the DQ-type I collagen channel.

Proximity ligation assay. MTLn3 cells were plated on gelatin matrix, fixed, permeabilized, and stained with primary antibodies for cortactin and pSerine and Phalloidin 488.

The proximity ligation assay was performed according to manufacturer's instructions for the Duolink II Probe anti-rabbit PLUS, PLA probe anti-mouse MINUS, and detection reagent orange (Olink Bioscience).

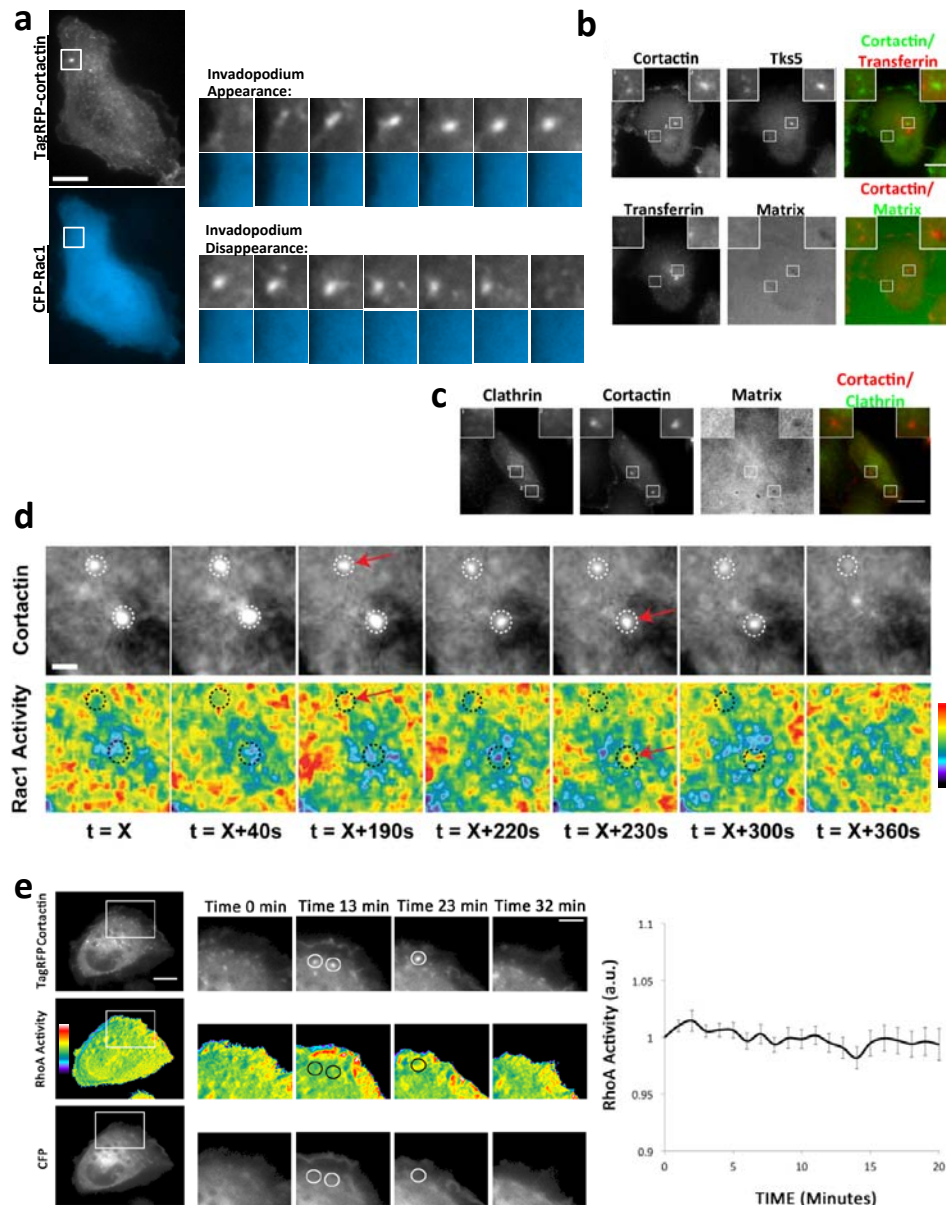
Statistical analysis. All *P* values were determined using a Student *t*-test. No statistical methods were used to pre-determine the sample size; no vertebrate animals were involved. No randomizations were used. The investigators were not blinded to allocation during experiments and outcome assessment. Statistical tests used are stated in every figure legend with *P* values as appropriate. Data distribution should meet the normal distribution requirements. No estimate of variation.

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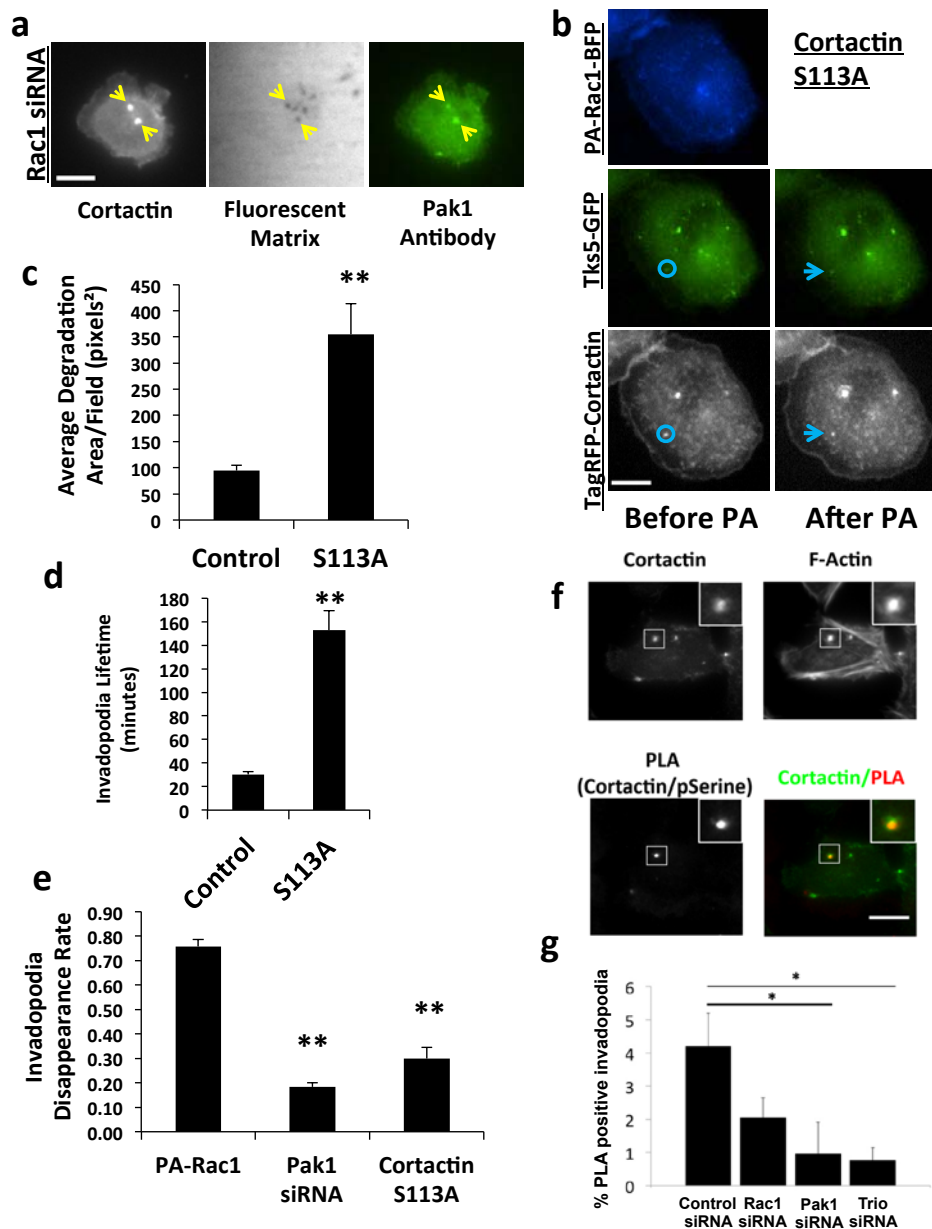
Supplementary Figure 1 Trio-Rac1-Pak1-axis promote invasion in human invasive tumor cells but not in normal breast epithelial cells, and RNAi specificity. (A) Representative images from human MDA-MB-231 showing cortactin (invadopodia marker) and changes in 405-gelatin matrix-degradation with siRNA against: control, Rac1, Pak1, and Trio. Scale Bar= 10µm. Representative image set is from 40 image sets for Control, from 40 image sets for Rac1 siRNA, from 39 image sets for Pak1 siRNA, and from 41 image sets for Trio siRNA. (B) Left: Quantification of the degradation area per field in MDA-MB-231 from (A). n= 39 fields per condition, pooled from 3 independent experiments. Student's t-test: ***p=3.18x10⁻⁶(Rac1), ***p=1.67x10⁻⁵(Pak1), ***p=2.81x10⁻⁶(Trio). Right: MDA-MB-231 with control, Rac1, Pak1 and Trio-siRNA show reduced invasion through matrigel. n=24 fields per condition, pooled from 6 independent experiments. Student's t-test: ***p=6x10⁻⁵(Rac1), ***p=6.9x10⁻⁵(Pak1), **p=0.005(Trio). (C) Representative images from human BT-549 showing cortactin (invadopodia marker) and changes in 405-gelatin matrix-degradation with siRNA against: control, Rac1, Pak1, and Trio. Scale Bar= 10µm. Representative image set is from 29 image sets for Control, from 28 image sets for Rac1 siRNA, from 35 image sets for Pak1 siRNA, and from 31 image sets for Trio siRNA. (D) Left: Quantification of the degradation area per field in BT-549 from (C). n= 29 fields per condition, pooled from 3 independent experiments. Student's t-test: *p=0.042(Rac1), *p=0.048(Pak1), **p=0.008(Trio). Right: BT-549 with control, Rac1, Pak1 and Trio-siRNA show reduced invasion through matrigel. n= 16 fields pooled from 4 independent experiments per condition. Student's t-test: *p=0.01(Rac1), **p=0.009(Pak1), *p=0.02(Trio). (E)

Representative images from human MCF10A showing cortactin and the 405-gelatin matrix with siRNA against: control, Rac1, Pak1, and Trio. Scale Bar= 10µm. Representative image set is from 40 image sets for Control, from 40 image sets for Rac1 siRNA, from 43 image sets for Pak1 siRNA, and from 40 image sets for Trio siRNA. (F) Left: Quantification of the degradation area per field in MCF10A from (E). n= 40 fields per condition, pooled from 3 independent experiments. Student's t-test: p= 0.3(Rac1), p= 0.7(Pak1), p=0.7(Trio). Right: Invasion through matrigel in MCF10A with control, Rac1, Pak1 and Trio-siRNA. n=24 fields per condition, pooled from 6 independent experiments. Student's t-test: p=0.4(Rac1), p=0.059(Pak1), p=0.09(Trio). (G) Top: Western blot of Rac1, Pak1, Trio, β-actin, mDia1, GAPDH levels in control, Rac1, Pak1, and Trio-siRNA-treated MDA-MB-231. Middle: Western blot of Rac1, Pak1, Trio, β-actin, mDia1 levels in control, Rac1, Pak1, and Trio-siRNA-treated BT-549. Bottom: Western blot of Rac1, Pak1, Trio, mDia1, GAPDH levels in control, Rac1, Pak1, and Trio-siRNA-treated MCF10A. All blots shown are from single blots. (H) Western blots from MTLn3 with control and single oligonucleotides of Rac1 (top), Pak1 (middle) or TrioGEF (bottom)-siRNA and detecting Rac1, β-actin (top), Pak1, β-actin (middle) or Trio, mDia1 (bottom). Right: Quantifications of matrix-degradation for single oligonucleotide-knockdown condition. n=29 fields for each condition, pooled from 3 independent experiments, with S.E.M. Student's t-test: ***p=1.8x10⁻¹⁷(#2); p=5.7x10⁻¹²(#4:Rac1-siRNA), ***p=0.0001(#1); ***p=8.7x10⁻⁸(#3:Pak1-siRNA), ***p=5.6x10⁻⁷(#1); ***p=9.4x10⁻⁶(#3:TrioGEF-siRNA). Representative blots are shown from 1 blot for Rac1 panel, 1 blot for Pak1 panel and 2 blots for the TrioGEF panel.



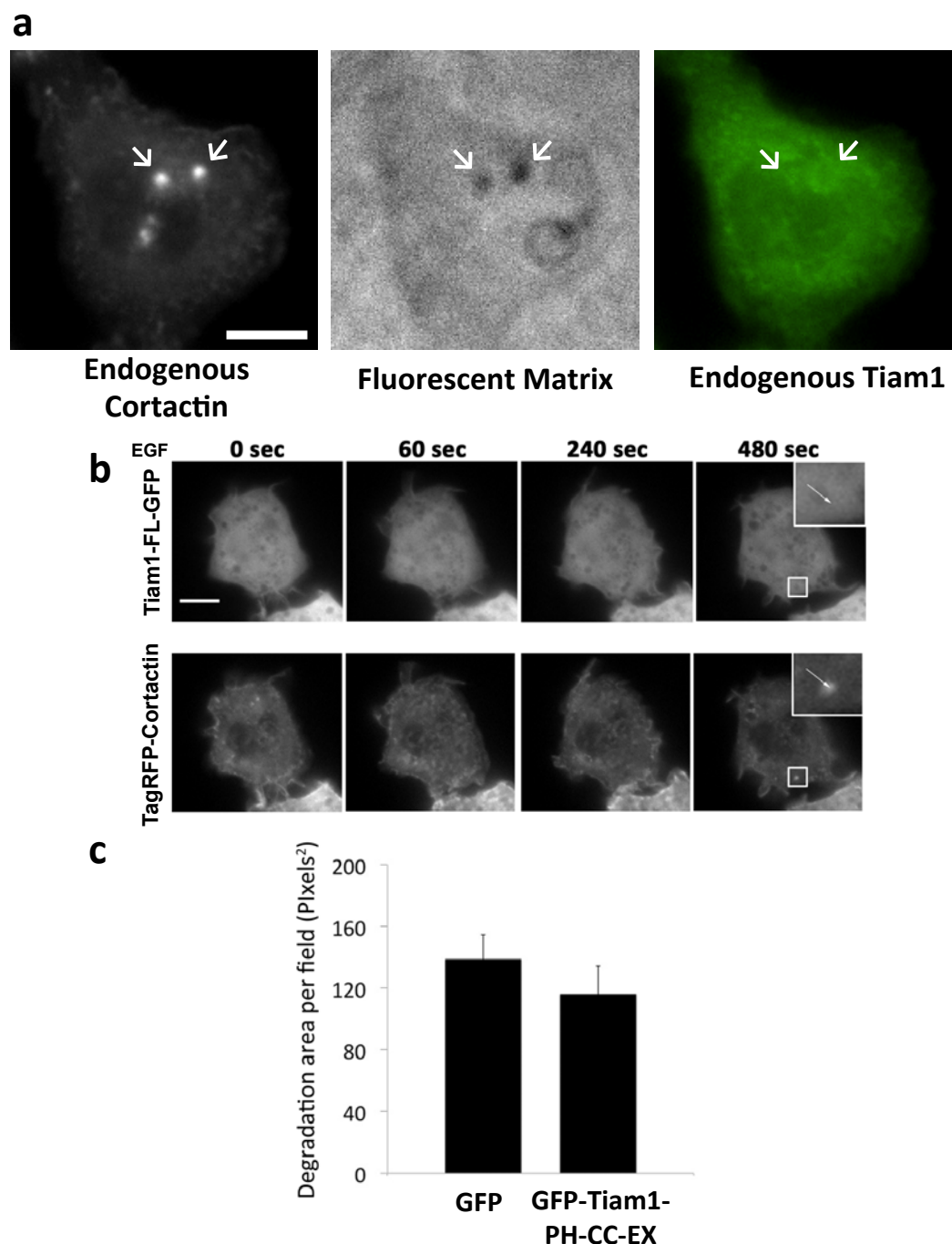
Supplementary Figure 2 Rac1 is inactive in, but does not accumulate at mature invadopodia, the structures are non-vesicular, Rac1 activity in disappearing invadopodia, and control RhoA biosensor at steady-state invadopodia. (A) Still-frames taken from a live-cell movie of MTLn3 expressing TagRFP-cortactin and CFP-Rac1. Insets show zoom on a single invadopodium (white box) during formation up to steady-state (top) and before and during disassembly (bottom). Throughout the invadopodium's lifetime Rac1 does not accumulate at the cortactin spot, and maintains its homogeneous distribution. Scale bar=10 μ m. (B) Representative image of MTLn3 plated on 405-gelatin matrix and stained for Transferrin, cortactin and TKS5. Insets show magnified invadopodium precursor with no matrix degradation activity (left white box), and a mature invadopodium actively degrading the gelatin matrix (right white box). In both cases there is no enrichment of the vesicle marker transferrin. Scale bars=10 μ m. (C) Representative image of an MTLn3 cell plated on 405-gelatin matrix and stained with clathrin and cortactin antibodies to show endogenous localization. Scale Bar= 10 μ m. Insets show magnified invadopodium precursor with no matrix degradation activity (left white box), and a mature invadopodium

actively degrading the gelatin matrix (right white box). In both cases there is no enrichment of the vesicle marker clathrin. (D) Additional examples of Rac1 activity changes at invadopodia prior to the disappearance. White and black circles show the invadopodium location and corresponding Rac1 activity levels. Red arrows indicate the moment of elevated Rac1 activity prior to the disappearance of invadopodium. Pseudocolor scale: 1.0 (Black) to 1.62 (Red). Scale bar=2 μ m. (E) Left: RhoA activity dynamics in RhoA biosensor-expressing MTLn3 cells, transfected with TagRFP-cortactin and imaged under steady state conditions on gelatin matrix. Images were acquired every minute. TagRFP-cortactin (top), RhoA activity (middle) and biosensor localization (bottom) images are shown at different time points. White boxes are magnified on the right. Black and white circles indicate invadopodia. Scale bar=10 μ m and 5 μ m (insets). Pseudocolor scale: 1.0 (Black) to 3.0 (White). Right: Quantification of RhoA activity (normalized to time=0) during steady-state invadopodia lifetimes. No significant change in RhoA activity was observed between any of the time points ($p>0.05$). $n=16$ invadopodia, pooled from 3 independent experiments, with S.E.M. Student's t-test was used.



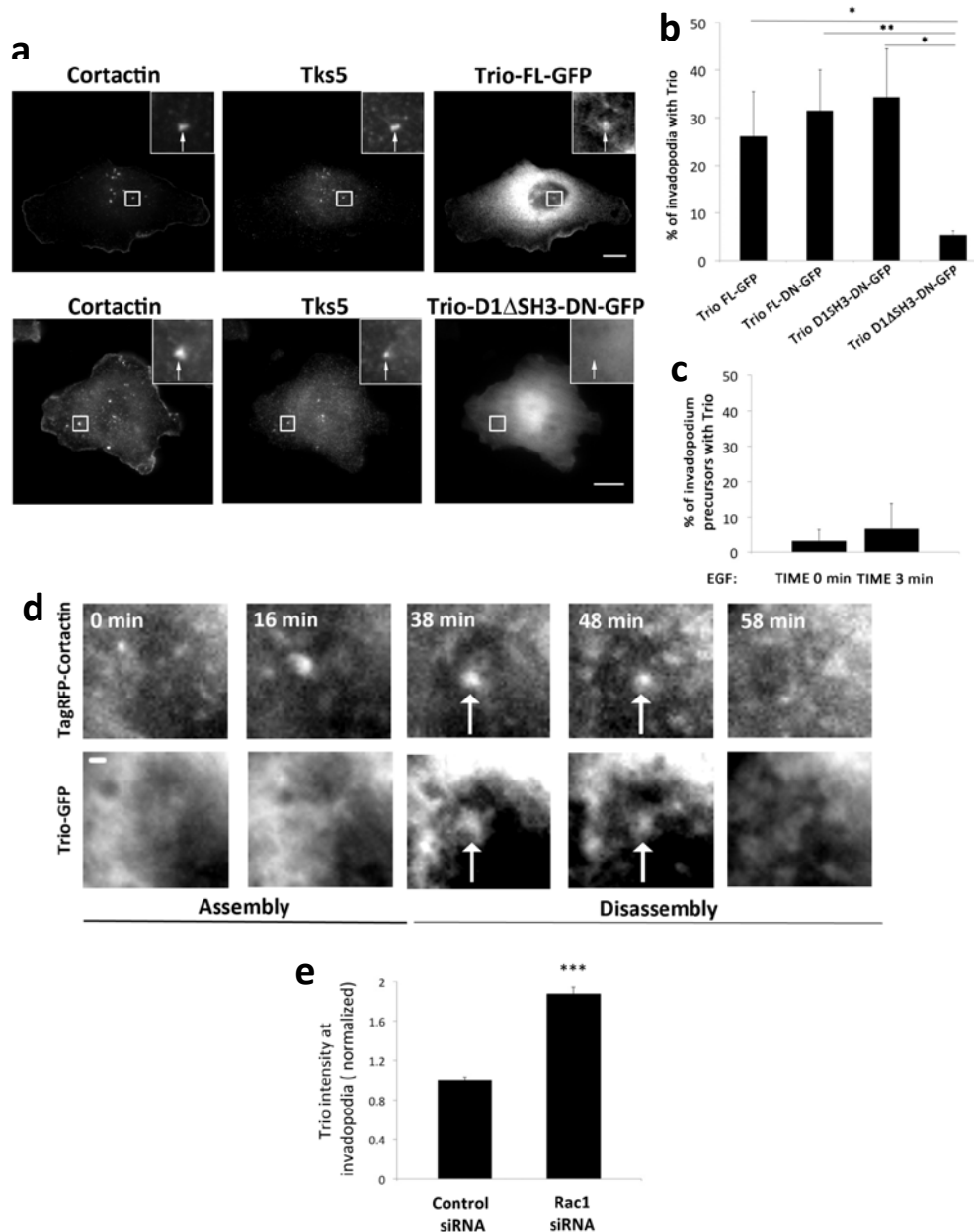
Supplementary Figure 3 Pak1 is recruited to invadopodia in Rac1-depleted cells, photo-uncaging of Rac1 at invadopodia containing phosphorylation deficient cortactin, and Pak1 modulates serine phosphorylation of cortactin, (A) Representative image of MTLn3 cell transfected with Rac1 siRNA, then plated on Alexa-405 conjugated gelatin and fixed and stained with cortactin and Pak1 antibodies to show endogenous protein localization. Arrows point to sites of mature, degrading invadopodia. Scale bar=10µm. Representative image set is from 30 image sets. (B) Representative images of MTLn3 cell transfected with PA-Rac1, S113A TagRFP-cortactin, and Tks5-GFP before and after photoactivation. Blue circles show photoactivated region. Blue arrows point to invadopodia after photoactivation. Scale bars=10µm. Representative image set is shown from 51 image sets. (C) Expression of cortactin S113A mutant alone increases matrix degradation area. Quantification of mean degradation area of control and cortactin mutant-overexpressing cells plated on fluorescent matrix overnight. n=30 fields, pooled from 3 independent experiments, with S.E.M. Student's t-test was used. **p=3.7571x10⁻⁵. (D) Quantification of mean invadopodia lifetime in control and cortactin S113A overexpressing cells, from time-lapse movies over a period of 6 hours. Invadopodia lifetime was quantified as the time in minutes from the appearance to the disappearance of RFP-cortactin and

Tks5-GFP co-localized spots. n=11 invadopodia for each condition, pooled from 3 independent experiments, error bars are S.E.M. Student's t-test was used. **p=9.193x10⁻¹³. (E) Quantification of percentage of invadopodia that disappear as a result of photoactivation with PA-Rac1, in control, Pak1 knockdown, and cortactin S113A mutant conditions. n=46 invadopodia (PA-Rac) pooled from 3 independent experiments, n=27 invadopodia (Pak1 siRNA) pooled from 3 independent experiments, n=61 invadopodia (Cortactin S113A) pooled from 3 independent experiments, error bars are S.E.M. Student's t-test was used. **p=3.278x10⁻⁵ (Pak1 siRNA), **p=1.91167x10⁻⁵ (Cortactin S113A). (F) Representative images of MTLn3 cell fixed and stained with antibodies to cortactin, pSerine, and 488-phalloidin (F-actin) showing: Proximity ligation assay spots (cortactin-phosphoserine interaction), cortactin and F-actin. Insets show sites of colocalization of cortactin with F-actin, and cortactin with the phosphoserine-cortactin interaction detected by PLA. Scale bar=10µm. Representative image set is from 46 image sets. (G) Quantification of the percentage of invadopodia containing positive PLA staining for control, Rac1, Pak1, and TrioGEF-knockdowns. Invadopodia were identified as F-actin and cortactin positive spots. n=300 invadopodia per condition, pooled from 3 independent experiments, error bars are S.E.M. Student's t-test was used. *p=0.0397 (Pak1 KD), *p=0.019 (Trio KD).



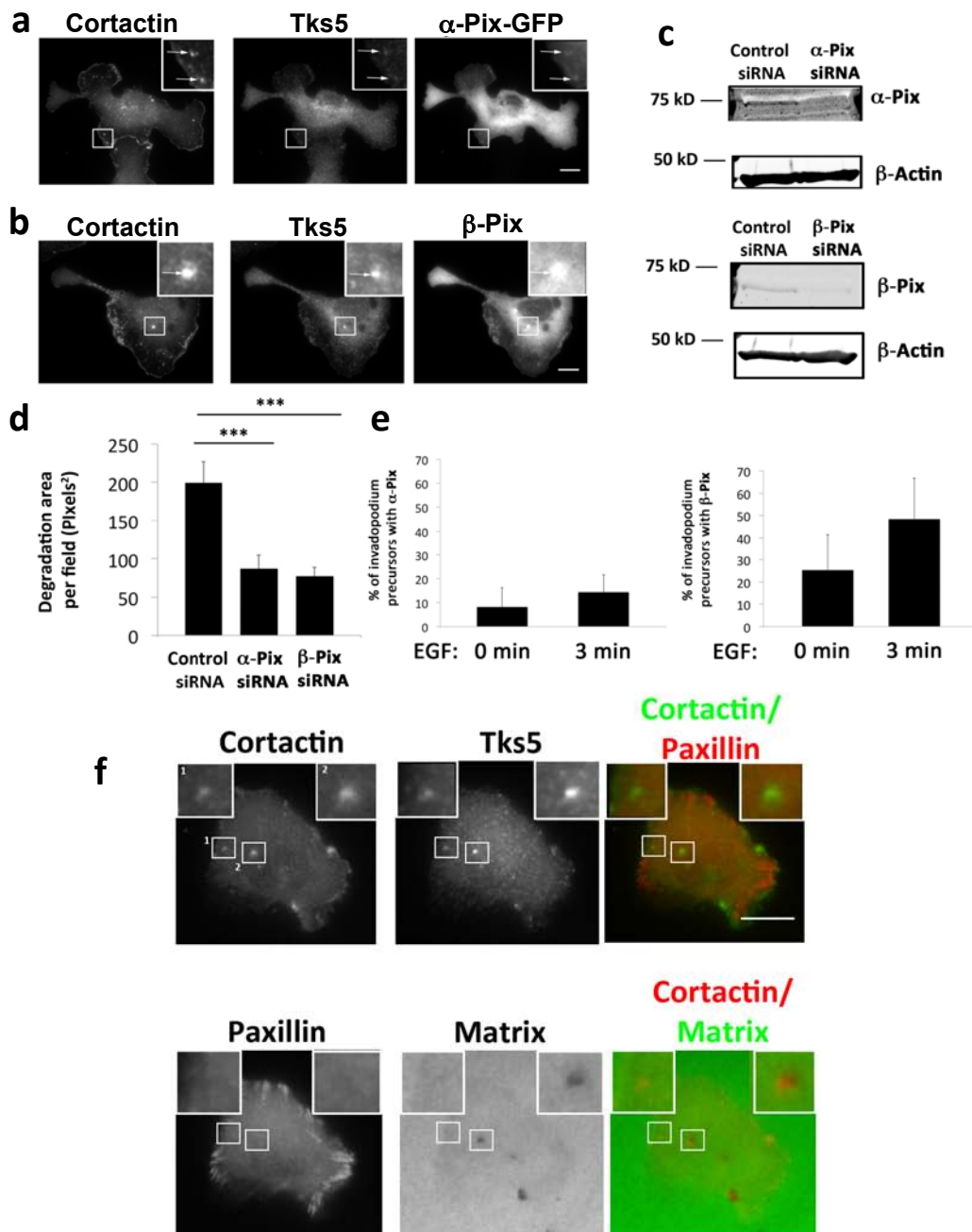
Supplementary Figure 4 Tiam1 does not accumulate at invadopodia and is not required for matrix degradation activity. (A) MTLn3 cell plated on Alexa-405 conjugated gelatin matrix and fixed and stained with antibodies for cortactin and Tiam1. Arrows point to sites of mature invadopodia. Endogenous Tiam1 is uniformly distributed and does not focally accumulate at invadopodia. Scale Bar= 10µm. Representative image set is from 30 image sets. (B) Still images from a time lapse movie of MTLn3 cell transfected with Tiam1-FL-GFP (FL: Full-Length) and TagRFP-cortactin, and

starved and stimulated with EGF. Insets show an invadopodium precursor formed after stimulation (bottom panel), while there is no corresponding enrichment of Tiam1 (top panel). Scale bar = 10µm. Representative image set is from 16 cell movies. (C) Quantification of average matrix degradation area per field from MTLn3 cells transfected with GFP or a dominant negative Tiam1 mutant (GFP-Tiam1-PH-CC-EX) and plated overnight on fluorescent matrix. n=31 fields for each condition, pooled from 3 independent experiments, error bars are S.E.M. Student's t-test was used. p=0.347.



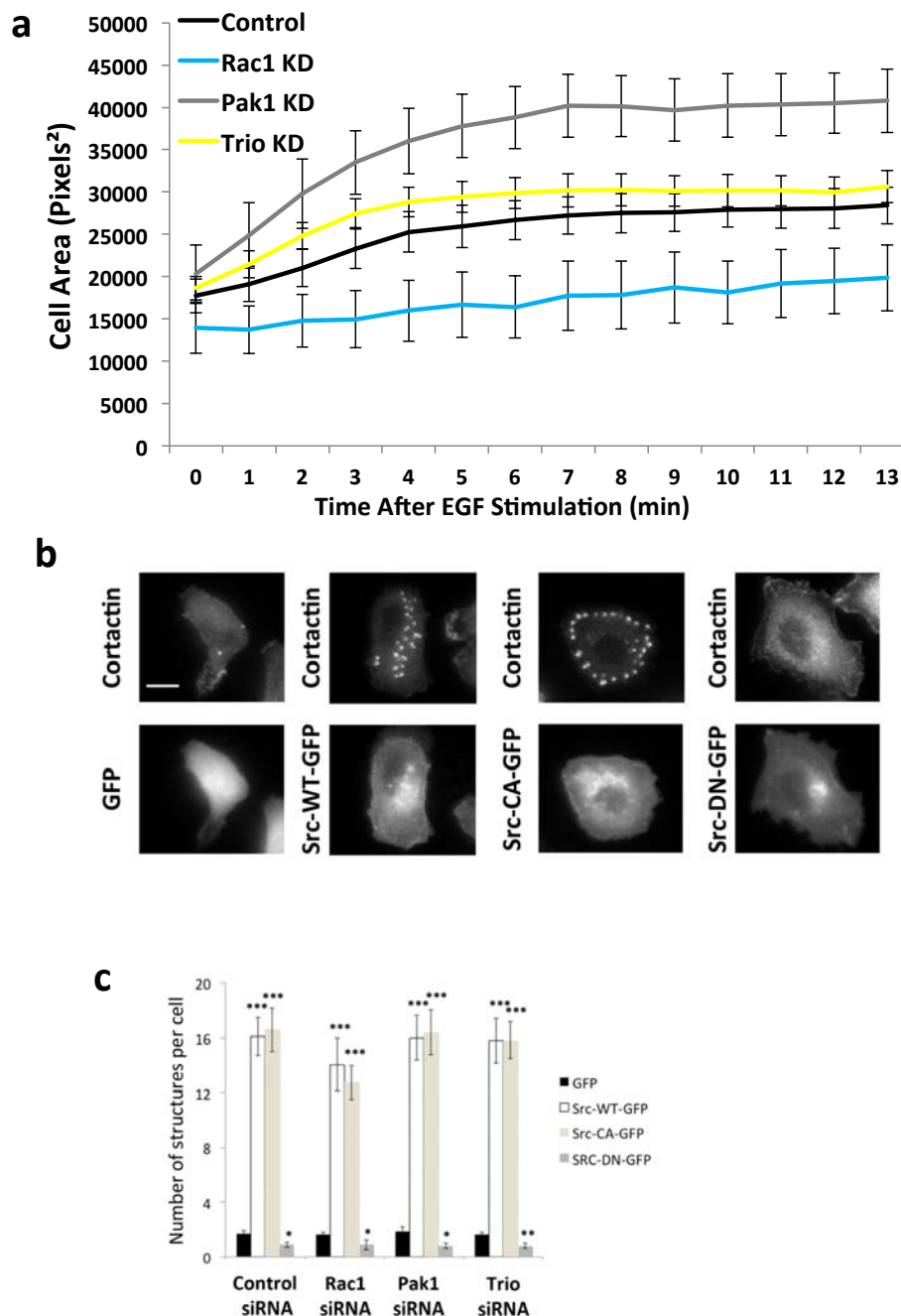
Supplementary Figure 5 TrioGEF recruitment and regulation at invadopodia. (A) Representative images of MTLn3 cells transfected with Trio-FL-GFP (FL: Full-Length) or Trio-D1ΔSH3-DN-GFP (D1SH3 fragment lacking the SH3 domain and with a point mutation at N1406A-D1407A making it dominant negative (DN)). Cells were fixed and stained with cortactin and TKS5 antibodies. White boxes are magnifications of the indicated areas. Arrows point to single invadopodia. Scale bar= 10μm. (B) Quantification of the percentage of invadopodia that show Trio at invadopodia. The bars represent the percentage of Trio positive invadopodia. n=100 invadopodia per condition, pooled from 5 independent experiments, error bars are S.E.M. Student's t-test was used. *p=0.031 (Trio-FL-GFP vs. Trio-D1ΔSH3-DN-GFP),

p=0.0075 (Trio-FL-DN-GFP vs Trio-D1ΔSH3-DN-GFP), *p=0.011 (Trio-D1SH3-DN-GFP vs. Trio-D1ΔSH3-DN-GFP). (C) Quantification of the percentage of invadopodia that show Trio at invadopodia before and after EGF stimulation. n=30 invadopodia per condition, pooled from 3 independent experiments, error bars are S.E.M. Student's t-test was used. p=0.6. (D) Examples of Trio recruitment to invadopodium prior to invadopodium disappearance. MTLn3 cells were transfected with cortactin-mRFP and Trio-GFP and image every 2 minutes for 3 hours. Scale bar= 1μm. (E) Trio recruitment to invadopodia in MTLn3 cells treated with control or Rac1 siRNA. n=150 invadopodia, pooled from 3 independent experiments, with S.E.M. Student's t-test was used. *p=2.25x10⁻³⁴.



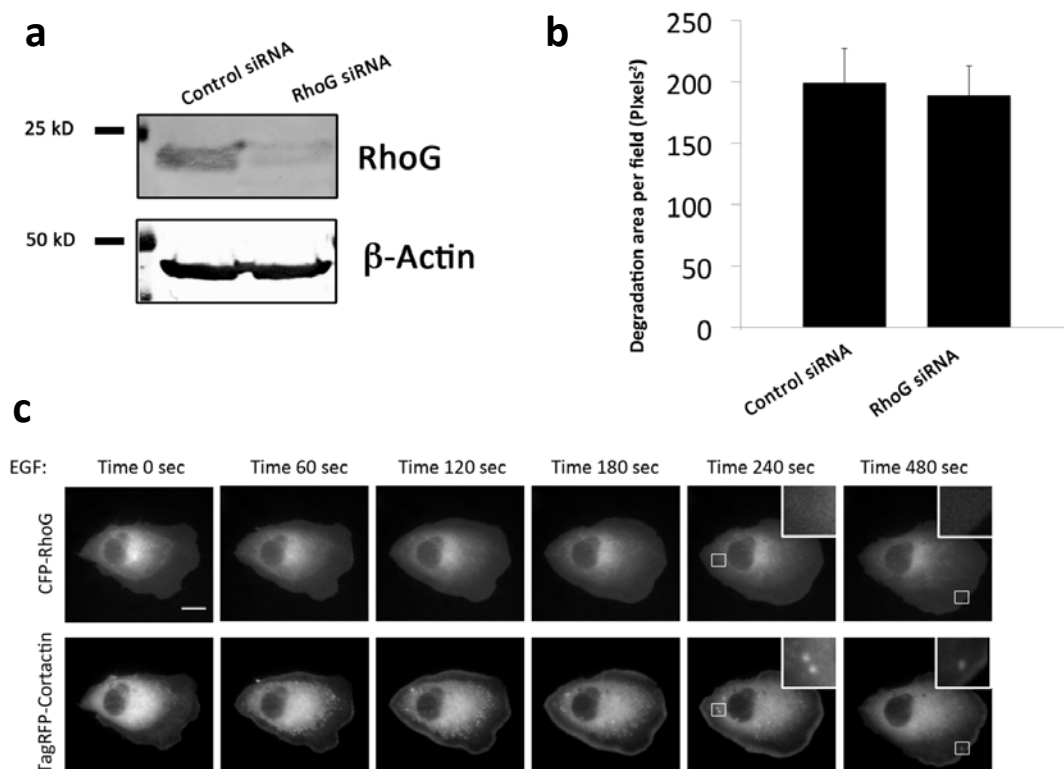
Supplementary Figure 6 Depletion of α -Pix or β -Pix inhibits matrix degradation by invadopodia and paxillin does not accumulate at invadopodia. (A) Representative images of MTLn3 cell transfected with α -Pix-GFP and stained for cortactin and Tks5 as invadopodia markers. White square boxes are magnified. Arrows point to invadopodia. Scale bar = 10 μ m. Representative image set is from 9 image sets. (B) Representative images of MTLn3 cell transfected with β -Pix-GFP and stained for cortactin and Tks5 as invadopodia markers. White square boxes are magnified. Arrows point to invadopodia. Scale bar = 10 μ m. Representative image set is from 13 image sets. (C) Western blot of MTLn3 cells transfected with α -Pix, β -Pix or control siRNA and blotted for α -Pix, β -Pix, and β -actin. Representative blot from 1 blot each for the α - and β -Pix. (D) Quantification of the degradation area per field in MTLn3 cells transfected with control, α -Pix,

or β -Pix siRNA. $n=40$ fields per condition, pooled from 4 independent experiments, with S.E.M. Student's t-test was used. *** $p=0.0004$ (α -Pix), *** $p=3.37 \times 10^{-5}$ (β -Pix). (E) Quantification of percentage of invadopodia with α -Pix (left) or β -Pix (right) before and after EGF stimulation for 3 minutes. $n=40$ invadopodia for each condition, pooled from 3 independent experiments, with S.E.M. Student's t-test was used. $p=0.59$ (α -Pix), $p=0.39$ (β -Pix). (F) Representative image of an MTLn3 cell plated on Alexa-405 conjugated gelatin matrix and fixed and stained with paxillin, cortactin and Tks5 antibodies. White squares show magnifications of an invadopodium precursor with no matrix degradation activity (left), and a mature invadopodia actively degrading the gelatin matrix. No enrichment of paxillin is seen at the core of the invadopodia in both cases. Scale bar = 10 μ m. Representative image set is from 10 image sets.



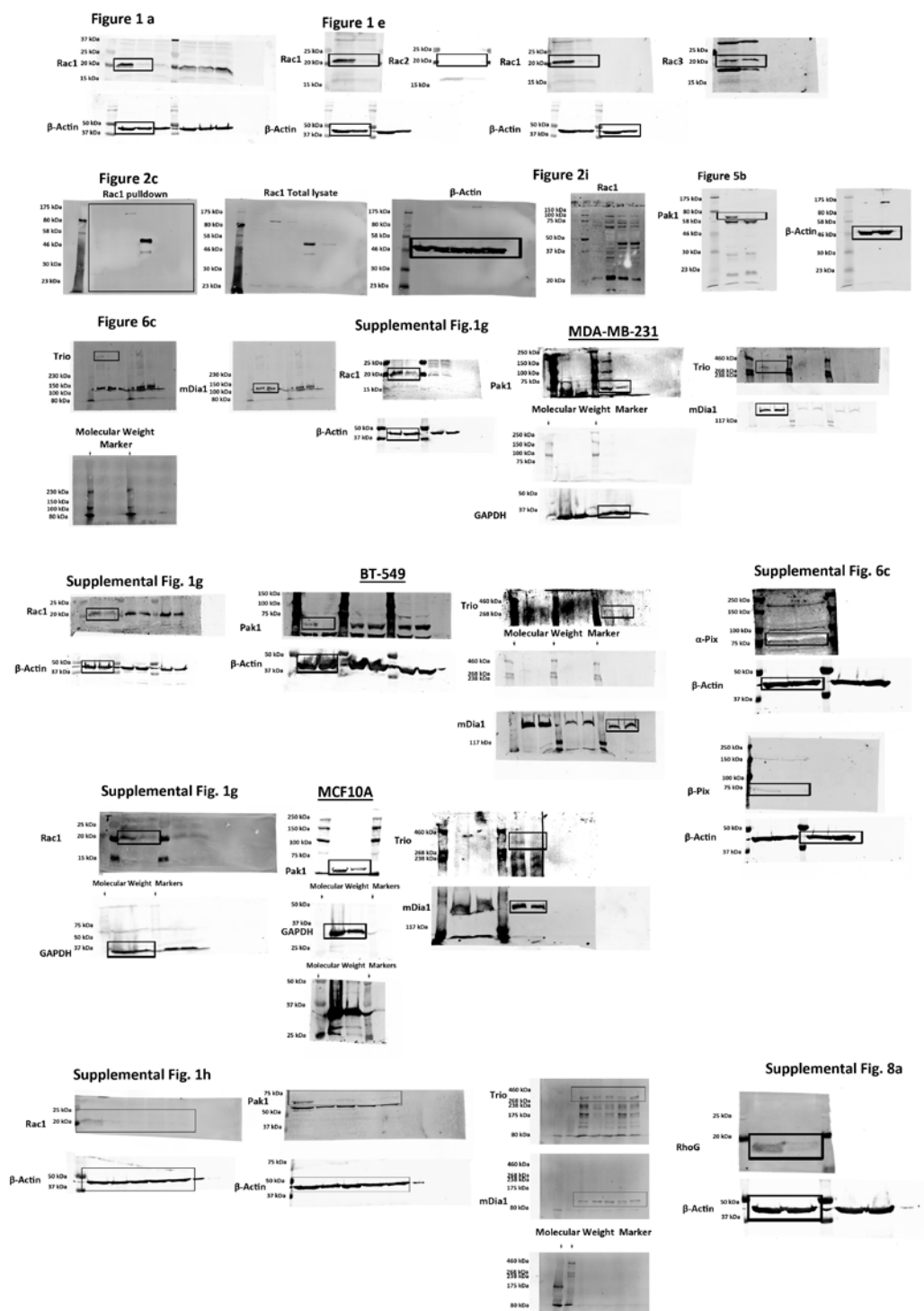
Supplementary Figure 7 Pak1 and Trio are not required for EGF-stimulated lamellipodia protrusion but Rac1 is required, and Src regulation of cortactin-Tks5-containing structures is independent of Trio-Rac1-Pak1-axis. (A) MTLn3 cells transfected with control, Rac1, Pak1 or Trio siRNA. Mean cell area over time is plotted for serum-starved MTLn3 cells stimulated with 5nM EGF, n=10 cells each from one experiment were analyzed, with S.E.M. Student's t-test was used. $p=5.06493 \times 10^{-08}$ (Rac1 KD), $p=1.29769 \times 10^{-05}$ (Pak1 KD), $p=0.058908959$ (Trio KD). (B) Representative images of MTLn3 cell transfected with GFP, wildtype c-Src-GFP (Src-WT-GFP), constitutively activated (Y528F; Src-CA-GFP) or dominant negative (K296R-Y528F; Src-DN-GFP). Cortactin staining is shown. Scale bar = 10µm. Representative image set is from 44 image sets for Cortactin-GFP-control, from 53 image

sets for Cortactin-Src-WT-GFP, from 43 image sets for Cortactin-Src-CA-GFP, and 47 image sets for Cortactin-Src-DN-GFP. (C) Quantification of the average number of cortactin and Tks5 positive structures per cell in MTLn3 cells treated with control, Rac1, Pak1 or Trio siRNA and transfected with GFP, Src-WT-GFP, Src-CA-GFP or Src-DN-GFP. n= 20 cells per condition, pooled from 7 independent experiments, error bars are S.E.M. Student's t-test was used. Control siRNA p-values: *** $p=3.3 \times 10^{-15}$ (Src-WT), *** $p=1.28 \times 10^{-14}$ (Src-CA), * $p=0.011$ (Src-DN). Rac1 siRNA p-values: *** $p=7.38 \times 10^{-18}$ (Src-WT), *** $p=6.84 \times 10^{-25}$ (Src-CA), * $p=0.035$ (Src-DN). Pak1 siRNA p-values: *** $p=2.35 \times 10^{-10}$ (Src-WT), *** $p=3.16 \times 10^{-12}$ (Src-CA), * $p=0.001$ (Src-DN). Trio siRNA p-values: *** $p=6.87 \times 10^{-12}$ (Src-WT), *** $p=4.08 \times 10^{-16}$ (Src-CA), * $p=0.003$ (Src-DN).



Supplemental Figure 8 RhoG is not required for invadopodia matrix degradation activity. (A) Western blot of RhoG and β -actin protein levels in control and RhoG siRNA treated cells. (B) Quantification of average degradation area per field in control and RhoG siRNA treated cells. $n=40$ fields per condition, pooled from 4 independent experiments, error bars are

S.E.M. Student's t-test was used. $p=0.77$. (C) Representatives images from a time lapse movie of MTLn3 cell transfected with cortactin-mRFP and RhoG-CFP, serum starved then stimulated with EGF for 8 minutes. White boxes are magnifications showing no enrichment of RhoG at invadopodia. Scale bar=10 μ m.



Supplemental Figure 9 Uncropped images of western blots. Black box indicated the cropped part included in the corresponding figure.

Supplemental Video Legends**Supplemental Video 1**

Invadopodia lifetimes are dramatically increased when Rac1 is silenced. Rac1-depleted MTLn3 cell transfected with two invadopodia markers, TagRFP-cortactin and TKS5-GFP, and time-lapse imaged every 2 minutes over 6 hours. Cortactin and TKS5 fluorescence co-localization show invadopodia.

Supplemental Video 2

Rac1 activity at invadopodia is abrogated during invadopodium formation. Serum-starved MTLn3 cell expressing Rac1 biosensor and TagRFP-cortactin stimulated with EGF (zoomed view of single invadopodium). Imaged every 10 seconds for 10 minutes.

Supplemental Video 3

Rac1 activity inside the invadopodium is significantly lower compared to the region outside, until when the invadopodium disappears. MTLn3 cell expressing Rac1 biosensor and TagRFP-cortactin at steady state (zoomed view of single invadopodium). Imaged every 1 minute.

Supplemental Video 4

Focal photo-uncaging of PA-Rac1 induces disappearance of invadopodium. Uncaging Rac1 at a single invadopodium (arrow) in MTLn3 cell expressing PA-Rac1 and TagRFP-cortactin. Imaged every 5 seconds for 10 minutes. Photoactivation pulses start at 25 second time point.

Supplemental Video 5

Photo-uncaging the dark-mutant (C450A) of PA-Rac1 has no effect on invadopodium. Photoactivation of single invadopodium (arrow) using the C450A light-insensitive PA-Rac1 mutant in an MTLn3 cell expressing TagRFP-cortactin. Imaged every 5 seconds for 10 minutes. Photoactivation pulses start at 25 second time point.

Supplemental Video 6

Depletion of PAK1 prevents disappearance of invadopodium upon focal photo-uncaging of PA-Rac1. Uncaging Rac1 at a single invadopodium (arrow) in an MTLn3 cell treated with PAK1 siRNA and expressing TagRFP-cortactin. Imaged every 5 seconds for 10 minutes. Photoactivation pulses start at 25 second time point.

Supplemental Video 7

Phosphorylation deficient mutant (S113A) of cortactin prevents disappearance of invadopodium upon focal photo-uncaging of PA-Rac1. Uncaging Rac1 at a single invadopodium (arrow) in a cell overexpressing S113A-mutated TagRFP-cortactin. Imaged every 5 seconds for 10 minutes. Photoactivation pulses start at 25 second time point.

Supplemental Video 8

Invadopodia turnover dynamics under control conditions in MTLn3 cells. MTLn3 control cell transfected with two invadopodia markers, TagRFP-cortactin and TKS5-GFP, and time lapse-imaged every 2 minutes over 6 hours. Cortactin and TKS5 fluorescence co-localization show invadopodia.

Supplemental Video 9

Invadopodia turnover dynamics in MTLn3 cells treated with Rac1 inhibitor NSC23766. MTLn3 cell transfected with two invadopodia markers, TagRFP-cortactin and TKS5-GFP, and time-lapse imaged every 2 minutes over 6 hours, with 100μM NSC23766 (Rac1 Inhibitor) treatment.

Supplemental Video 10

Invadopodia turnover dynamics in MTLn3 cells treated with Rac1 inhibitor Z62954982. MTLn3 cell transfected with two invadopodia markers, TagRFP-cortactin and TKS5-GFP, and time-lapse imaged every 2 minutes over 6 hours, with 25μM Z62954982 (Rac Inhibitor II) treatment.