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The Rac activator STEF (Tiam2) regulates cell migration by microtubule-mediated focal adhesion disassembly

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Focal adhesion (FA) disassembly required for optimal cell migration is mediated by microtubules (MTs); targeting of FAs by MTs coincides with their disassembly. Regrowth of MTs, induced by removal of the MT destabilizer nocodazole, activates the Rho-like GTPase Rac, concomitant with FA disassembly. Here, we show that the Rac guanine nucleotide exchange factor (GEF) Sif and Tiam1-like exchange factor (STEF) is responsible for Rac activation during MT regrowth. Importantly, STEF is required for multiple targeting of FAs by MTs. As a result, FAs in STEF-knockdown cells have a reduced disassembly rate and are consequently enlarged. This leads to reduced speed of migration. Together, these findings suggest a new role for STEF in FA disassembly and cell migration through MT-mediated mechanisms.

Keywords: focal adhesions; microtubules; Rac; STEF; Tiam2

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INTRODUCTION

During processes such as embryogenesis, immune response and wound healing, the orchestrated movement of cells in a particular direction is essential and highly regulated, integrating signals controlling adhesion, polarity and the cytoskeleton. Deregulation of migration can lead to the formation of tumours and metastases. The small Rho-like GTPases Rac1, RhoA and Cdc42 control cell adhesion and migration through modulation of both actin and microtubule (MT) cytoskeletons (Watanabe *et al*, 2005; Ridley, 2006). Guanine nucleotide exchange factors (GEFs), which are activators of Rho GTPases, are also emerging as crucial regulators of these processes. The Rac-GEF T-lymphoma invasion and metastasis 1 (Tiam1) regulates cell migration through modulation of both cell–cell and cell–substrate adhesion (Woodcock *et al*, 2009). However, its closest family member Sif and Tiam1-like exchange factor (STEF; Hoshino *et al*, 1999) has not yet been implicated in these processes.

Assembly and disassembly of focal adhesions (FAs) are crucial processes during cell migration. These multi-molecular complexes attach the intracellular cytoskeleton to the extracellular matrix (Horwitz & Webb, 2003). So far, more than 150 components of FAs have been identified (Zaidel-Bar *et al*, 2007). Determining the mechanisms that regulate their formation and disassembly is crucial to furthering our understanding of cell migration. Whereas the mechanisms of FA disassembly are not completely understood, a clear role has emerged for FA kinase (FAK). *FAK*-deficient cells have large FAs owing to their impaired disassembly (Ilic *et al*, 1995; Webb *et al*, 2004). Furthermore, Src, extracellular signal-related kinase (ERK) and myosin light chain (MLC) kinase have been implicated owing to their ability to rescue the impaired FA disassembly of *FAK*-deficient cells (Webb *et al*, 2004). Rho GTPases also regulate FA disassembly. FAK/Src signalling can phosphorylate and activate p190RhoGAP, decreasing Rho activity and reducing adhesion-associated stress fibres, which are dependent on the Rho effector ROCK. ROCK inhibition decreases FA size (Arthur *et al*, 2000; Schober *et al*, 2007). However, other signalling pathways must be involved, as inhibition of ROCK alone was not sufficient to restore FA disassembly rates in *FAK*-deficient fibroblasts (Webb *et al*, 2004).

The Rac effector p21-activated kinase (PAK) has also been implicated in FA disassembly. PAK localizes to focal complexes (Manser *et al*, 1997) where it phosphorylates the integral FA component paxillin, resulting in adhesion turnover (Nayal *et al*, 2006). Furthermore, PAK phosphorylates MLC kinase, resulting in its inactivation (Sanders *et al*, 1999). As myosin-mediated contractility is required for both FA assembly (Chrzanowska-Wodnicka

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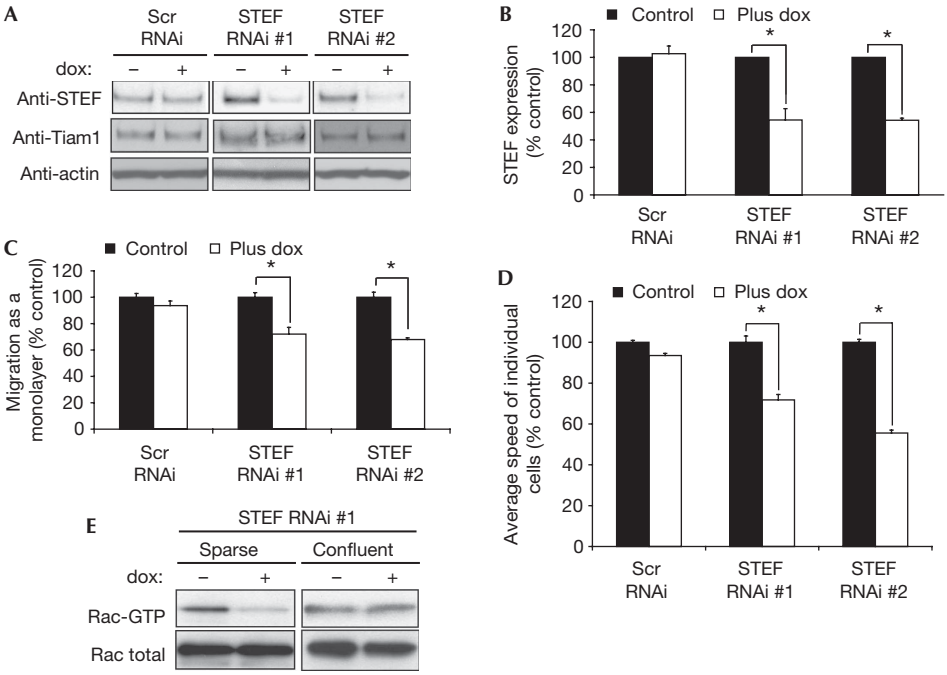

Fig 1 | STEF is required for optimal cell migration. (**A**) STEF and Tiam1 protein levels in ScrRNAi, STEF RNAi #1 and STEF RNAi #2 cells treated with or without dox. Actin was a loading control. (**B**) Mean STEF protein levels from three or more independent experiments (± s.e.m.); *$P<0.01$, *t*-test. (**C**) A confluent monolayer of cells cultured in the presence (plus dox) or absence (control) of dox was wounded and the area of the wound was measured immediately after wounding and 24 h later. The percentage of wounds closed is presented for cells expressing the various shRNAs and is shown as the mean (± s.e.m.) of three independent experiments; *$P<0.01$, *t*-test. (**D**) The average speed of migrating single cells cultured in the presence or absence of dox ± s.e.m.; *$P<0.01$, *t*-test. (**E**) Rac activity in cells in the presence or absence of dox, plated sparsely or at confluence. The data shown are representative of three independent experiments. dox, doxycycline; RNAi, RNA interference; shRNA, short hairpin interfering RNA; STEF, Sif and Tiam1-like exchange factor; Tiam1, T-lymphoma invasion and metastasis 1.

& Burridge, 1996; Nayal *et al*, 2006) and disassembly (Crowley & Horwitz, 1995), tight regulation of actomyosin contractility is important for FA turnover.

MTs have also emerged as important regulators of FA disassembly. They target FAs and multiple targeting of FAs by MTs leads to the disassembly of FAs (Kaverina *et al*, 1998, 1999). However, the pathways involved in the targeting of FAs by MTs, and how this results in their disassembly, are poorly characterized. Recently, the spectraplakin ACF7 was identified as a molecule that crosslinks MTs and actin filaments, guiding MTs towards FAs before their disassembly (Wu *et al*, 2008). Additionally, dominant-negative dynamin inhibits MT-induced FA disassembly, suggesting that MTs can induce disassembly by dynamin-driven endocytosis. Disassembly driven in this way depends on a dynamin–FAK interaction (Ezratty *et al*, 2005), although FAK itself is not required for MT targeting of FAs (Schober *et al*, 2007).

There is increasing evidence of crosstalk between MTs and Rho GTPases. MT growth activates Rac (Waterman-Storer *et al*, 1999). Furthermore, Tiam1 regulates MT stability (Pegtel *et al*, 2007). In this study, we identify STEF as a new regulator of FA disassembly and show that it regulates both MT-dependent Rac activation and MT targeting of FAs. Consequently, inhibition of the STEF/Rac signalling module leads to impaired FA disassembly, with a concomitant reduction in cell migration.

RESULTS AND DISCUSSION

STEF is required for optimal cell migration

To ascertain whether STEF has a role in cell migration, we used a doxycycline (dox)-inducible system to downregulate STEF in the skin papilloma cell line P1. This system encodes a short hairpin interfering RNA (shRNA), induced by the addition of dox (supplementary Fig S1 online), targeting transcripts for degradation by RNA interference (RNAi). STEF downregulation was assessed in cells expressing two different shRNA oligonucleotides—RNAi #1 and #2—and in control cells expressing a scrambled, non-targeting shRNA (Scr RNAi). The addition of dox to cells containing either STEF shRNA construct resulted in reduced STEF protein levels but did not affect STEF levels in scrambled control cells (Fig 1A,B). Tiam1 levels were unaffected by either STEF shRNA construct (Fig 1A).

The effect of STEF downregulation on cell migration was assessed by using a wound-healing assay. STEF-downregulated cells (plus dox) showed significantly reduced cell migration, closing on average 23% less of the wound as compared with control cells (Fig 1C). Furthermore, time-lapse analysis of sparsely plated cells showed that the average migration speed of individual STEF-downregulated cells was reduced by 30–45% (Fig 1D). To assess if STEF downregulation affects Rac activation in migrating cells, active Rac levels were determined in sparsely plated cells. Rac activity was reduced in STEF-downregulated

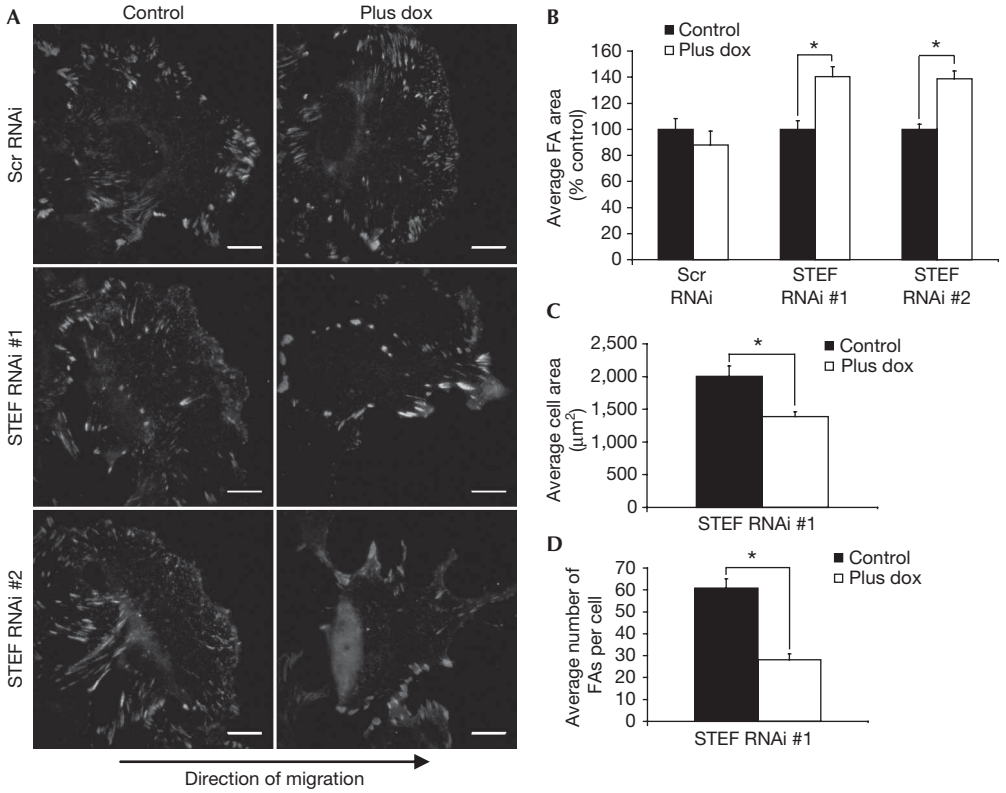

Fig 2 | Downregulation of STEF induces large focal adhesions. (**A**) Representative leading edge cells in the presence (plus dox) or absence (control) of dox immunostained with anti-vinculin to label FAs. Scale bars, 12 μm. (**B–D**) The average FA area of individual adhesions (**B**), the average cell area (**C**) and the average number of FAs per cell (**D**) in the control and STEF-downregulated cells. The data are presented as the mean (± s.e.m.) of 30 cells from three independent experiments; *$P<0.05$, *t*-test. dox, doxycycline; FA, focal adhesion; RNAi, RNA interference; STEF, Sif and Tiam1-like exchange factor.

cells (Fig 1E). However, when cells were plated at confluence, and therefore unable to migrate, no difference was observed (Fig 1E). Together, these data indicate that STEF is required for optimal migration of both wounded cell monolayers and individual P1 cells, and regulates the level of active Rac in migrating cells.

Rac activation by STEF controls FA size

A crucial element of cell migration is FA-mediated attachment to the substrate. There is increasing evidence of Rac involvement in FA regulation (Nayal *et al*, 2006; Chang *et al*, 2007). Hence, we considered that STEF downregulation results in deregulation of FAs, leading to the observed impaired migration. Immunostaining of migrating (leading edge) cells for a representative FA component, vinculin, showed that FAs in STEF-downregulated cells were larger than those in controls (Fig 2A). In both cases, vinculin-positive FAs were connected to actin stress fibres (supplementary Fig S2 online). Quantification—which excluded vinculin-positive small, dot-like focal complexes—showed that STEF downregulation induced a significant increase (40%) in the FA area (Fig 2B). Besides the increased FA area, the average cell area was significantly decreased (around 25%; Fig 2C), and the average number of FAs per cell was reduced by 50% (Fig 2D) in STEF-downregulated cells. Knockdown of Tiam1 in P1 cells did not affect the FA area (supplementary Fig S3 online).

We next tested whether the FA phenotype was due to the Rac-GEF activity of STEF. Previously, two point mutations in Tiam1 were shown to abolish its Rac-GEF activity (Tolias *et al*, 2005); therefore, we mutated the corresponding residues in STEF to alanine. GEF-mutant STEF (EGFP-ΔN-STEF-GEF*) was unable to activate Rac (Fig 3A). Furthermore, expression of this mutant in P1 cells led to increased FA area (Fig 3B,C), comparable to the effects of STEF downregulation in P1 cells, suggesting that the GEF-mutant STEF is dominant-negative. By contrast, expression of an amino-terminally truncated form of STEF (ΔN-STEF), known to be constitutively active (Hoshino *et al*, 1999), increased active Rac levels (Fig 3A) and reduced the FA area (Fig 3B,C). Together, these data indicate that STEF regulates the size of FAs through Rac.

STEF regulates FA disassembly

The increased size of FAs led us to consider that FA disassembly kinetics might be reduced after STEF downregulation. Control or STEF-downregulated P1 cells at the wound edge were micro-injected with paxillin-GFP (paxillin is an integral FA protein), and analysed subsequently by fluorescence time-lapse microscopy. The adhesions of control cells completely disassembled in the timeframe of the experiment, whereas they persisted in STEF-downregulated cells (Fig 4A,B). The rates of FA disassembly of individual adhesions were determined. The average rate constant for disassembly of adhesions in control cells was

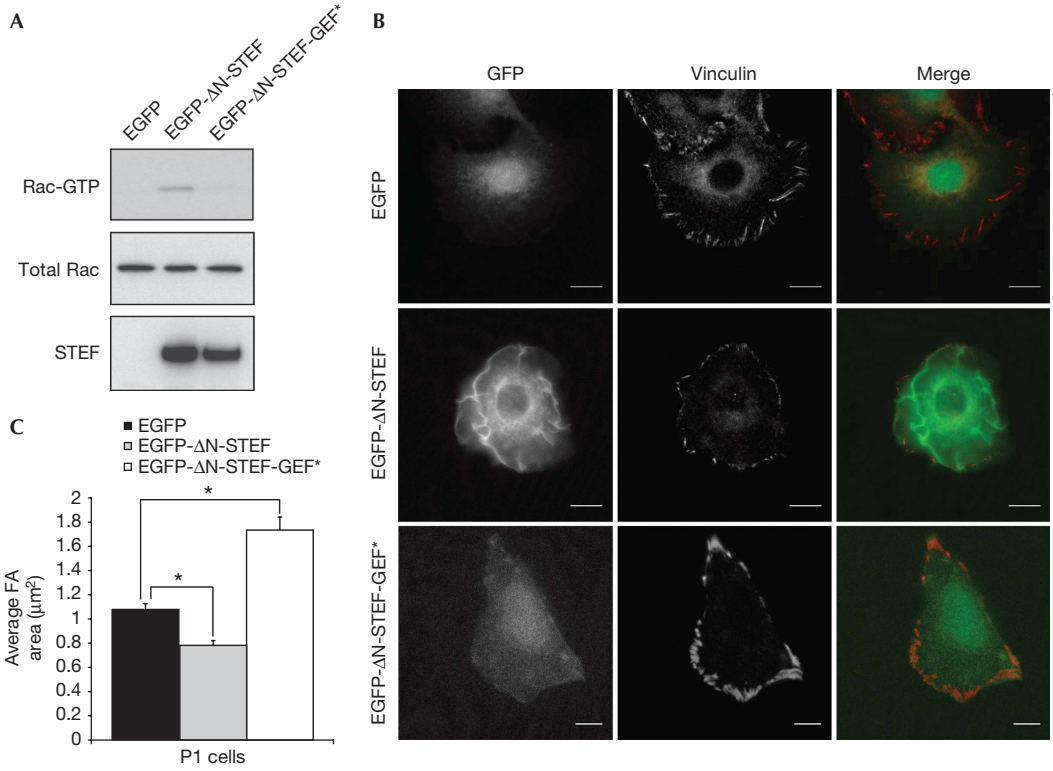

Fig 3 | Rac activation by STEF controls focal adhesion size. (**A**) Rac activity was measured in Cos-7 cells transfected with control EGFP, EGFP-ΔN-STEF or ΔN-STEF-GEF-mutant (EGFP-ΔN-STEF-GEF*) plasmids. (**B**) P1 cells transfected with the indicated constructs were immunostained with anti-vinculin. Scale bars, 12 μm. (**C**) The average FA area of P1 cells transfected with the indicated constructs. Data presented are the mean (± s.e.m.) of 30 cells from three independent experiments; $*P<0.001$, *t*-test. EGFP, enhanced green fluorescence protein; FA, focal adhesion; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; STEF, Sif and Tiam1-like exchange factor.

$(5.7 \pm 0.7) \times 10^{-2}\,\mathrm{min}^{-1}$, whereas in STEF-downregulated cells it was significantly lower at $(3.2 \pm 0.5) \times 10^{-2}\,\mathrm{min}^{-1}$ (Fig 4C). This suggests that the increased FA size observed in STEF-downregulated cells is the result of impaired FA disassembly. Furthermore, we infer that this underlies the defect in cell migration after STEF knockdown.

STEF levels do not regulate ERK, MLC kinase and RhoA

The regulation of FA disassembly is poorly understood. However, a few proteins, including ERK, MLC kinase and Rho, have been implicated (Webb *et al*, 2004). Therefore, we determined whether STEF/Rac signalling regulates these molecules in migrating cells. However, we did not detect any differences in ERK and MLC phosphorylation, or Rho activity, in STEF-downregulated cells plated sparsely (supplementary Fig S4A,B online). Increases in phospho-ERK, phospho-MLC and Rho activity were, however, detected in cells treated with the MT-disrupting agent nocodazole, which is known to induce large, stabilized FAs (Bershadsky *et al*, 1996; Liu *et al*, 1998) and was a positive control in these assays (supplementary Fig S4A,B online).

STEF mediates MT-dependent activation of Rac

Targeting of FAs by growing MTs coincides with their disassembly (Kaverina *et al*, 1998, 1999; Ezratty *et al*, 2005; Wu *et al*, 2008). Furthermore, MT growth, induced by nocodazole washout, activates Rac (Waterman-Storer *et al*, 1999). Additionally, Rac through PAK is implicated in FA turnover (Nayal *et al*, 2006). Given that in STEF-downregulated migrating cells we observed decreased Rac activity and impaired FA disassembly, we considered that STEF might mediate MT-dependent Rac activation. To test this, we performed nocodazole washout experiments followed by Rac activity measurements. As expected, in control cells we observed increased Rac activation after nocodazole washout (that is, MT regrowth); however, this was not apparent in STEF-downregulated cells (Fig 5A). In addition, expression of the GEF-mutant STEF in P1 cells impaired Rac activation in response to MT regrowth (Fig 5B).

To determine whether the effect of STEF downregulation on Rac activation is specific for MT-dependent Rac stimulation or a general defect, we induced Rac activation with epidermal growth factor (EGF) in P1 cells. Here, STEF-downregulated cells and control cells were equally capable of activating Rac and inducing membrane ruffling (supplementary Fig S5A,B online). This indicates that STEF-downregulated cells are not inherently defective in Rac activation. Together, these data show that STEF is required for Rac activation, through its Rac-GEF activity, specifically after MT regrowth.

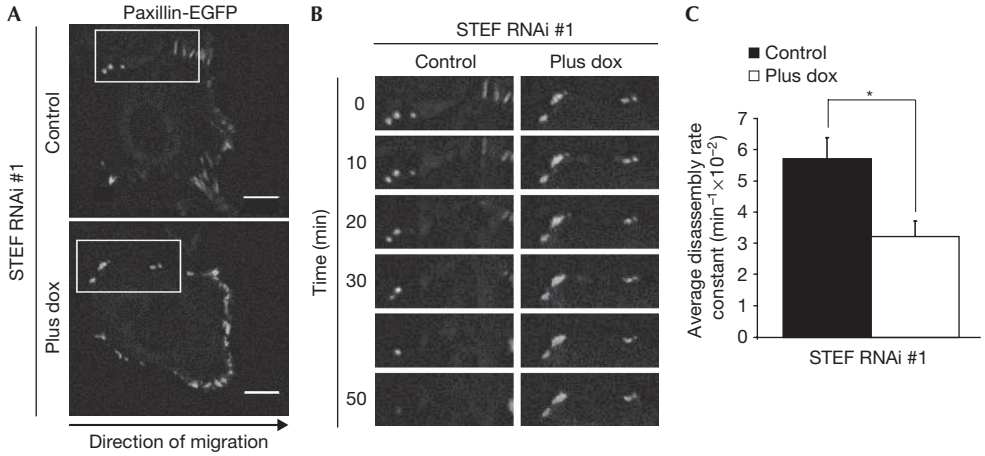

Fig 4 | STEF is required for focal adhesion disassembly. (**A–C**) Wound edge cells cultured in the presence (plus dox) or absence (control) of dox were microinjected with paxillin-EGFP. (**A**) Representative fluorescence images. (**B**) Boxed areas in (**A**) shown over time. (**C**) The average disassembly rate (min^{-1}) of FAs in control and STEF-downregulated cells. Data presented are the mean (± s.e.m.) of more than 20 individual FAs; $*P<0.005$, *t*-test. dox, doxycycline; EGFP, enhanced green fluorescence protein; FA, focal adhesion; RNAi, RNA interference; STEF, Sif and Tiam1-like exchange factor.

STEF regulates MT-mediated FA disassembly

MT regrowth, induced by nocodazole washout, reduces FA size (Ezratty *et al*, 2005). We therefore examined FA size during the nocodazole washout experiment. As anticipated, FA size increased in all cells treated with nocodazole (Fig 5C). Significantly, whereas washout of nocodazole (that is, MT regrowth) in control cells resulted in the expected diminution of FAs, in STEF-downregulated cells FAs remained stable (Fig 5C,D). MT regrowth was not affected by STEF downregulation (Fig 5C), indicating that STEF is required specifically for the process of FA disassembly after MT regrowth.

As the effects of STEF, with respect to Rac activity and FA regulation, are linked closely to MTs, we next examined whether STEF is required for optimal MT targeting of FAs, a process linked with their disassembly (Kaverina *et al*, 1998, 1999). To study MT targeting of FAs, we performed total internal reflection fluorescence microscopy on cells coexpressing pEGFP-β-tubulin (to label MTs) and mCherry-paxillin (to label FAs), as we have done previously (Krylyshkina *et al*, 2003). Time-lapse images show that whereas MTs target FAs in control cells, they fail to optimally target FAs in STEF-downregulated cells (Fig 5E). As multiple targeting events are associated with FA disassembly (Kaverina *et al*, 1998, 1999), the number of MT targeting events—defined as the extension of a MT into an adhesion site, so that the two can no longer be resolved—during a 10 min time-lapse video were quantified for individual FAs in control and STEF-downregulated cells. Overall, a significantly lower percentage of adhesions were targeted more than once in STEF-downregulated cells (40%) when compared with control cells (61%; Fig 5F). Together, these data indicate that STEF is required for multiple targeting of MTs to FAs, which in turn facilitates their disassembly.

In conclusion, we have shown that STEF regulates FA disassembly and consequently cell migration. We found that the FAs in STEF-downregulated cells were enlarged, reminiscent of those induced by nocodazole treatment (Bershadsky *et al*, 1996). We therefore examined the role of MTs downstream from STEF/Rac signalling in FA disassembly. It has been shown that active Rac promotes the growth of MTs into the lamellipodium by modulating the MT plus-tip protein Clasp2 (Wittmann *et al*, 2003; Wittmann & Waterman-Storer, 2005). It has also been shown that MT growth, induced by washout of nocodazole, activates Rac (Waterman-Storer *et al*, 1999). In conjunction with these previous findings, the data presented here support the idea that STEF/Rac signalling regulates the growth of MTs towards specific sites, such as FAs. Collectively, the data also suggest the presence of a positive-feedback loop in which MTs activate STEF to activate Rac. This active Rac then promotes the targeted growth of MTs towards FAs, hence perpetuating the Rac activation signal and the multiple-targeting of MTs at these specific sites. In terms of FA disassembly, localized activation of Rac would allow the recruitment of Rac effector(s)—such as PAK (Nayal *et al*, 2006)—to FAs, promoting their disassembly. It would be interesting in the future to determine the pathway downstream from STEF/Rac that leads to targeting and disassembly of FAs as well as the mechanism by which MTs activate the STEF/Rac module.

METHODS

Cell culture. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. shRNA-expressing cells were maintained in DMEM with 10% tetracycline-free fetal bovine serum and 400 μg/ml G418. The cells were transfected using Fugene6 (Roche Diagnostics, Burgess Hill, UK), and retrovirally transduced as described previously (Woodcock *et al*, 2009). For nocodazole washout experiments, cells were plated on glass-bottom dishes coated with fibronectin (10 μg/ml; Sigma, Poole, UK) and serum-starved for 24 h. Nocodazole (Sigma) was added at a final concentration of 10 μM for 4 h, washed out and cells were processed for immunofluorescence or lysed to assess Rac activity, as described in the supplementary information online.

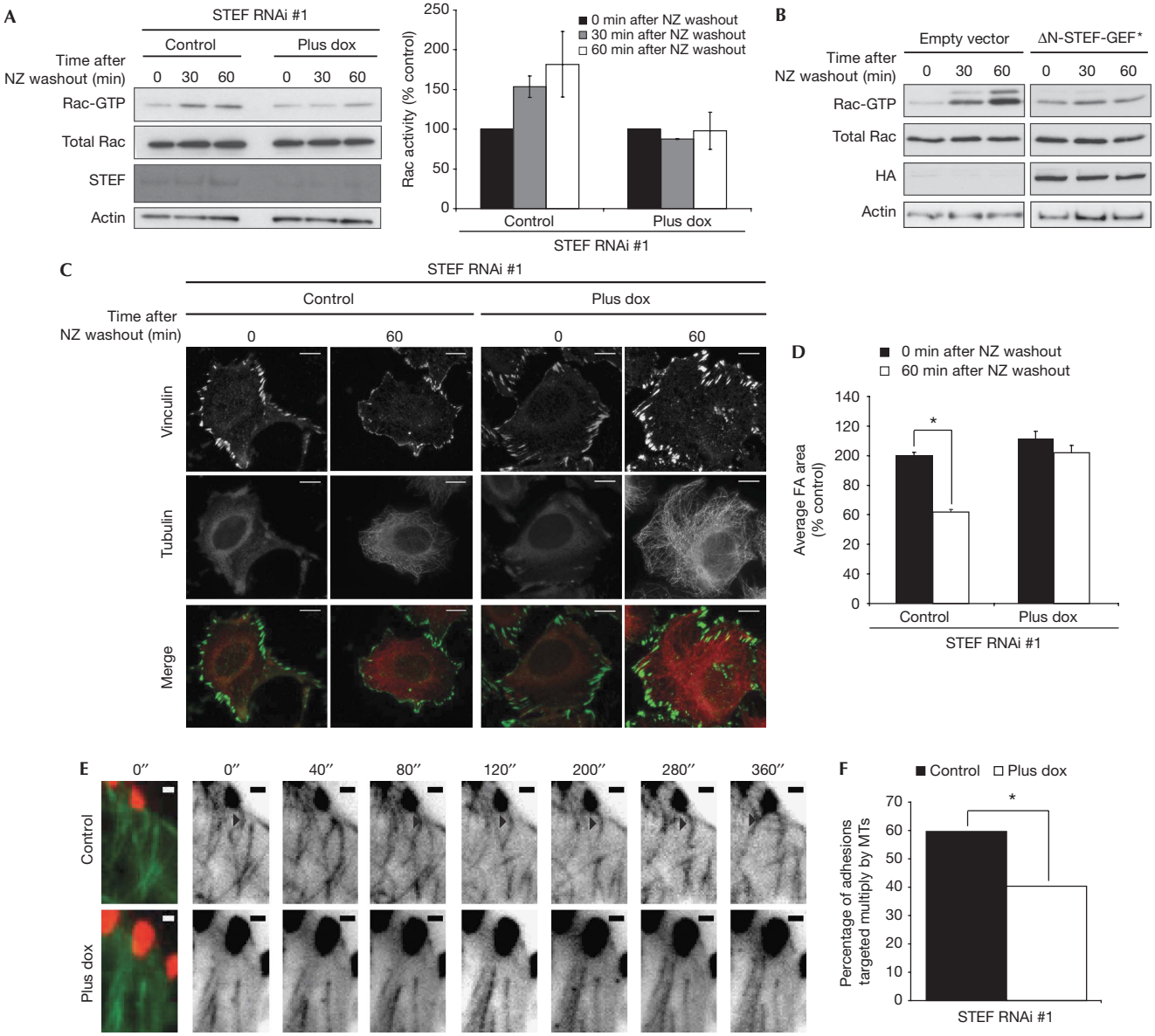

Fig 5 | STEF is required for microtubule-dependent activation of Rac and multiple targeting of focal adhesions by microtubules. (**A**) Cells in the presence (plus dox) or absence (control) of dox were treated with 10 μM nocodazole (NZ) for 4 h. Rac activity was measured 0, 30 and 60 min after NZ washout. Rac activity was quantified from three independent experiments. (**B**) P1 cells infected with either empty vector or GEF-mutant STEF (ΔN-STEF-GEF*) were treated as in (**A**) and Rac activity was measured. Anti-HA shows expression levels of GEF-mutant STEF. The data shown are representative of three experiments. (**C**) Cells were treated as in (**A**), fixed at the time points indicated and immunostained for vinculin and tubulin. Scale bars, 12 μm. (**D**) FA size was determined in control and STEF-downregulated cells at 0 and 60 min after NZ washout. Data presented are the mean (± s.e.m.) of 30 cells from three experiments; $*P<0.001$, *t*-test. (**E**) Wound edge cells were microinjected with mCherry-paxillin (red) and pEGFP-β-tubulin (green). Total internal reflection fluorescence microscopy time-lapse images were recorded at 5 s intervals for 10 min. Representative negative contrast images from control and STEF-downregulated cells, and a coloured image of the first time point in each case, are shown. MTs targeting FAs are indicated by the black arrows in the control time-lapse image. Scale bars, 1 μm. (**F**) FAs targeted more than once in the 10 min time-lapse period. $*P<0.05$, Mann–Whitney test. Data shown are the culmination of more than 50 adhesions from more than 20 cells. dox, doxycycline; FA, focal adhesion; GEF, guanine nucleotide exchange factor; HA, haemagglutinin; MT, microtubule; STEF, Sif and Tiam1-like exchange factor.

Antibodies. Immunoblotting and immunofluorescence were performed as described previously (Woodcock *et al*, 2009), using antibodies listed in the supplementary information online.

FA disassembly assay. Wound edge cells microinjected with pEGFP-paxillin were visualized with a time-lapse imaging system. The images were collected at 100 ms exposures for 3 h at 5 min

intervals. The intensity of individual adhesions was determined over time by using the ImageJ software (NIH, Bethesda, MD, USA) and disassembly rate was determined as previously described previously (Webb *et al*, 2004).

Total internal reflection fluorescence microscopy. Total internal reflection fluorescence microscopy to visualize targeting of FAs by MTs was performed as described previously (Krylyshkina *et al*, 2003); further details are available in the supplementary information online. From videos, targeting events were counted for several individual FAs.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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