Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton

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Regulation of the actin cytoskeleton by microtubules is mediated by the Rho family GTPases. However, the molecular mechanisms that link microtubule dynamics to Rho GTPases have not, as yet, been identified. Here we show that the Rho guanine nucleotide exchange factor (GEF)-H1 is regulated by an interaction with microtubules. GEF-H1 mutants that are deficient in microtubule binding have higher activity levels than microtubule–bound forms. These mutants also induce Rho-dependent changes in cell morphology and actin organization. Furthermore, drug-induced microtubule depolymerization induces changes in cell morphology and gene expression that are similar to the changes induced by the expression of active forms of GEF-H1. Furthermore, these effects are inhibited by dominant-negative versions of GEF-H1. Thus, GEF-H1 links changes in microtubule integrity to Rho-dependent regulation of the actin cytoskeleton.

Cell migration is important in many physiological processes, including embryonic development, wound healing and the immune response. Cell migration is powered by the activity of the actin cytoskeleton, with actin polymerization driving leading edge protrusion and acto-myosin contractility promoting cell body advancement. To support directional migration, actin dynamics and myosin contractility need to be precisely regulated in a spatially and temporally appropriate manner. Paradoxically, although microtubules do not directly contribute to the generation of forces that drive cell migration in most cell types, the loss of microtubules prevents directional movement of cells in culture[1], suggesting that microtubules may be involved in the regulation of actin-dependent motility. Indeed, fibroblasts lacking microtubules cannot form lamellipodia in a directional fashion and instead extend new membrane protrusions in a random manner over the cell periphery[1]. The rate of lamellipodial protrusion in these cells is decreased, suggesting that microtubules are necessary to support normal rates of leading edge protrusion[2]. Interestingly, a decrease in lamellipodial activity is also observed in cells treated with low concentrations of the microtubule-depolymerizing drug nocodazole, which inhibit the dynamics of microtubules without inducing complete depolymerization[3]. Thus, dynamic microtubules seem to be involved in promoting leading edge protrusion. In addition to regulating actin polymerization at the leading edge, microtubules also modulate actin filament organization and myosin contractility in the cell body, as microtubule disassembly promotes the formation of stress fibres and enhances contractility[4].

Recent studies indicate that regulation of the actin cytoskeleton by microtubules relies on the activity of Rho family GTPases (reviewed in ref. 5). Microtubule growth induces activation of Rac, which in turn promotes the formation of lamellipodia[6]. By contrast microtubule disassembly results in the activation of Rho, which enhances myosin contractility and stress fibre formation[7–9]. At present, the molecular mechanisms through which microtubules modulate the activity of Rho GTPases is unknown. However, the recent identification of GEFs that interact with microtubules[10–12] presents several candidates for the function of a microtubule-regulated Rho/Rac activator. These GEFs, murine Lfc and its human homologue GEF-H1, and p190RhoGEF belong to the Dbl family of Rho activators and contain the characteristic tandem arrangement of a Dbl homology (DH) domain and a pleckstrin homology (PH) domain. At present, there is no data regarding the regulation of the GEF activity of these proteins by microtubules. In the current study, we have analysed the function of microtubule binding in the regulation of GEF-H1 activity and provide experimental evidence that GEF-H1 is responsible for regulating Rho activity in response to microtubule depolymerization.

Results

Intracellular localization of GEF-H1. An earlier characterization of the intracellular distribution of GEF-H1 (amino acids 1–894) demonstrated that it localized to microtubules when expressed in cultured mammalian cells[10]. We cloned full-length GEF-H1 cDNA (see Methods) and found that, in common with GEF-H1(1–894), the full-length GEF-H1 protein also labelled microtubules (Fig. 1). Consistent with the previous study[10], we found that deletion of the entire carboxyl terminus of GEF-H1 resulted in the almost complete loss of microtubule localization (see Fig. 1b for an example of the typical cytosolic localization of the GEF-H1(1–572) construct). The GEF-H1-related KIAA-0651 protein (see Methods) was mostly diffusely distributed and did not localize to microtubules (data not shown). As KIAA-0651 differs from GEF-H1 primarily in its amino-terminal portion, we examined the effects of deletion of the N-terminal segment, including the zinc finger domain, from GEF-H1. The GEF-H1(92–985) construct did not label microtubules (data not shown), indicating that both C- and N-terminal regions of GEF-H1 are important for the interaction with microtubules. As these observations suggested that the N-terminal zinc finger domain could be involved in the interaction with microtubules, we

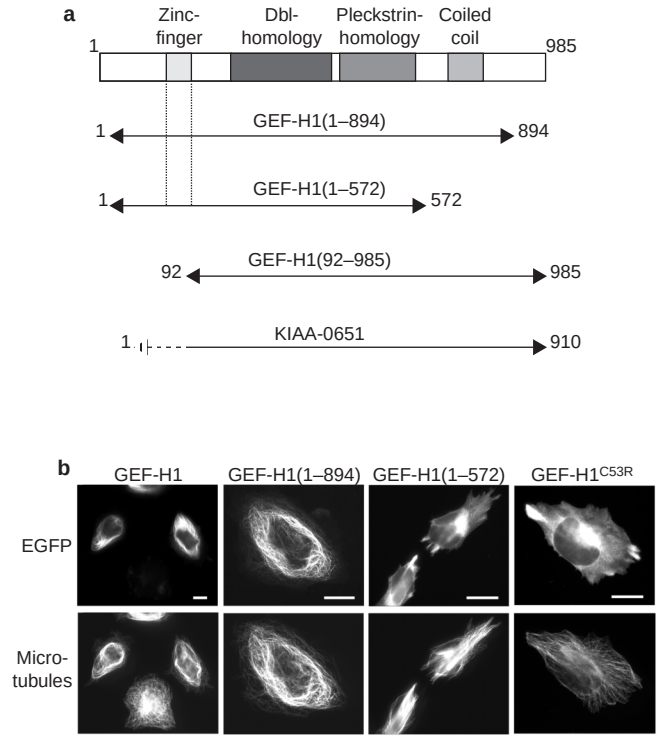

Figure 1 **GEF-H1 constructs and their localization. a,** A schematic representation showing the domain structure of the full-length GEF-H1 (amino acids 1–985) and the various expression constructs used in this study. The protein encoded by the KIAA-0651 cDNA clone is identical to GEF-H1 along most of its length (solid line) but differs from GEF-H1 at the N terminus (dashed line). **b,** The intracellular localization of GEF-H1 constructs. HeLa cells transfected with EGFP-tagged GEF-H1 constructs were fixed and stained with an anti-tubulin antibody. Full-length GEF-H1 and GEF-H1(1–894) colocalized with microtubules. GEF-H1(1–572) and GEF-H1^{C53R} (the zinc finger mutant) had a mostly diffuse cytoplasmic localization, with some enrichment at the tips of the projections of the cell edge. Scale bars represent 20 μm.

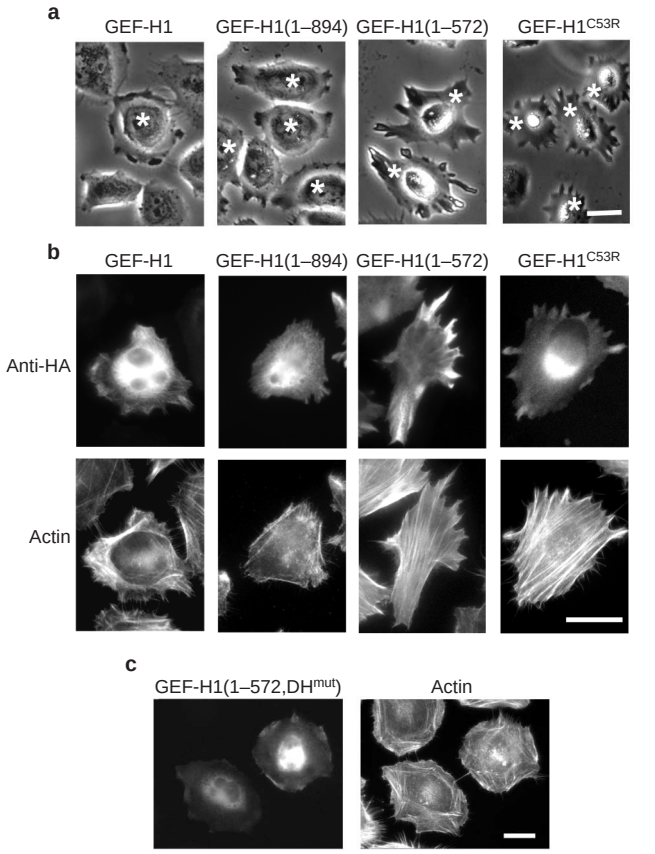

Figure 2 **Changes in cell morphology and actin organization induced by the expression of GEF-H1 constructs. a,** HeLa cells transfected with EGFP–GEF-H1 constructs were imaged by phase-contrast microscopy. Cells expressing GEF-H1 constructs (asterisks) were identified by EGFP fluorescence. Cells expressing full-length GEF-H1 and GEF-H1(1–894) had similar morphology to non-transfected cells, whereas cells expressing GEF-H1(1–572) and GEF-H1^{C53R} had an elongated, polarized shape and formed numerous projections along the edge. **b,** Transfected HeLa cells were fixed and stained with fluorescent phalloidin to label actin, and with anti-HA antibodies to label GEF-H1. Cells expressing GEF-H1(1–572) and GEF-H1^{C53R} exhibited more intense labelling of actin stress fibres than non-transfected cells or cells transfected with full-length GEF-H1 and GEF-H1(1–894). Stress fibres in GEF-H1(1–572)- and GEF-H1^{C53R}-expressing cells formed bundles extending into the projections of the cell edge. **c,** HeLa cells expressing HA-GEF-H1(1–572) with an inactivating mutation (Y393A) in the Dbl homology domain (DH mutant) were fixed and stained as in **b**. The actin organization of cells expressing GEF-H1(1–572,DH^{mut}) was similar to that of non-transfected cells. Scale bars represent 20 μm.

introduced an inactivating point mutation in the zinc finger (C53R)[13]. This mutation resulted in the loss of microtubule localization (see Fig. 1b).

Expression of enhanced green fluorescent protein (EGFP)–GEF-H1 or EGFP–GEF-H1(1–894) in HeLa cells resulted in the appearance of coiled microtubule bundles (Fig. 1b), which were reminiscent of the bundles of stable microtubules induced by overexpression of microtubule-associated proteins (MAPs)[14]. We examined whether overexpression of GEF-H1 resulted in increased microtubule stability. Bundled microtubules labelled with EGFP–GEF-H1 or EGFP–GEF-H1(1–894) were more resistant to nocodazole treatment than microtubules in control cells (data not shown). Additionally, microtubules in cells transfected with EGFP–GEF-H1 or EGFP–GEF-H1(1–894) contained high levels of acetylated α-tubulin, a post-translational modification typically found in stable microtubules[15]. Interestingly, expression of truncated GEF-H1 versions that do not localize to microtubules had no effect on microtubule stability. As GEF-H1 is a Rho GEF[10] (see also current study) and activation of Rho results in microtubule stabilization[16], we investigated whether the microtubule-stabilizing effects of GEF-H1 were mediated by a Rho signalling pathway. Inhibition of Rho with the dominant negative RhoA^{T19N} mutant, the Rhotekin Rho-binding domain (RBD) or the C3 exoenzyme had no effect on the ability of GEF-H1 to stabilize microtubules (data not shown). Thus, the stabilization of microtubules by GEF-H1 seems to be independent of Rho activation, and may be a result of the physical association of GEF-H1 with microtubules. In agreement with this hypothesis, a GEF-H1 point mutant that is deficient in nucleotide exchange activity (GEF-H1(DH^{mut}); see below) retained the ability to stabilize microtubules.

Effects of GEF-H1 on cell morphology and actin organization. While investigating the intracellular localization of various GEF-H1 constructs, we observed that transfection of HeLa cells with the versions of GEF-H1 deficient in microtubule binding induced dramatic changes in cell morphology (Fig. 2). HeLa cells expressing EGFP- or haemagglutinin (HA)-tagged GEF-H1(1–572), GEF-H1(92–985), KIAA-0651 or GEF-H1^{C53R} acquired an elongated, polarized shape with narrow, finger-like projections along the cell edge. Mutant GEF-H1 proteins were often enriched in the cell-edge projections (Fig. 2b). These projections were distinct in appearance from filopodia, the thin actin-rich membrane protrusions that can

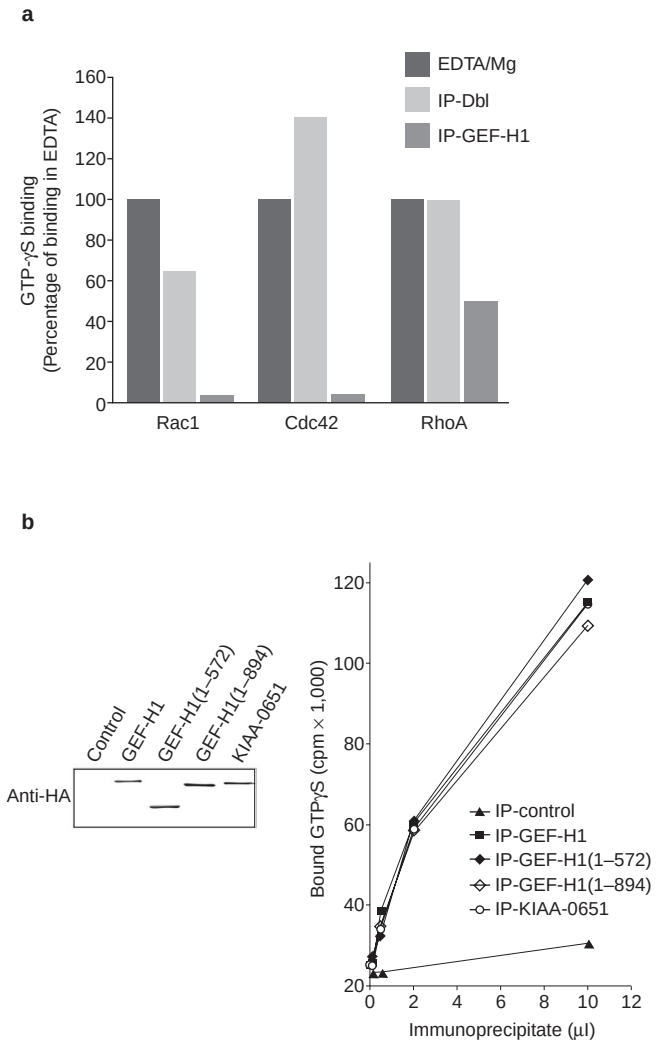

Figure 3 **The *in vitro* guanine nucleotide exchange activity of GEF-H1.** **a**, Oncogenic Dbl or full-length GEF-H1 immunoprecipitated from transfected Cos-1 cells were used to catalyse the binding of [^{35}S]GTP-γS to Rac1, Cdc42 or RhoA (see Methods). To promote forced nucleotide loading (EDTA/Mg), EDTA was added to a final concentration of 13.3 μM. Subsequently, nucleotide binding was stabilized by the addition of magnesium chloride to a final concentration of 30 mM. The background level of nucleotide binding observed in the absence of GEFs or EDTA was subtracted from all values and the level of nucleotide binding in the presence of EDTA was set to 100%. Although EDTA and Dbl promoted nucleotide exchange on all three GTPases studied, GEF-H1 catalysed nucleotide exchange only on RhoA. **b**, HA-tagged GEF-H1 constructs were immunoprecipitated from Cos-1 cells and used to catalyse nucleotide exchange on RhoA (right). All constructs were present in equivalent amounts in the immunoprecipitates (left) and showed similar exchange activity. Results shown in **a** and **b** are representative of at least two independent experiments.

be induced in HeLa cells by expression of constitutively active forms of Cdc42. Changes in overall cell morphology induced by mutant GEF-H1 constructs were accompanied by an increase in the number and intensity of actin stress fibres (Fig. 2b). In cells expressing mutant versions of GEF-H1, actin stress fibres often formed well-organized, parallel bundles that extended into the projections of the cell edge. Remarkably, expression of either full-length GEF-H1 or GEF-H1(1–894) had no noticeable effects on overall cell morphology or the organization of the actin cytoskeleton (Fig. 2a,b).

To determine whether the nucleotide exchange activity of GEF-H1 was required for its effects on cell morphology, we used site-directed mutagenesis to generate a Tyr to Ala amino-acid substitution at residue 393 in the conserved QRITKY sequence in the Dbl homology (DH) domain of GEF-H1 (Y393A). As with the analogous mutation in the DH domain of Lbc[17], this substitution completely abolished GEF-H1 nucleotide exchange activity *in vitro* (data not shown). Expression of truncated GEF-H1 constructs (GEF-H1(1–572) or KIAA-0651) containing the Y393A mutation had no effect on cell shape and actin organization (Fig. 2c), indicating that the ability to catalyse nucleotide exchange was crucial for the morphological effects of short versions of GEF-H1. This observation suggests that the morphological effects of GEF-H1 constructs were mediated by activation of the Rho family GTPases.

To identify specific Rho GTPases responsible for the cytoskeletal effects of GEF-H1 expression, we cotransfected HeLa cells with GEF-H1 constructs and the p21-binding domains (PBDs) of Rhotekin (an effector of Rho) or Pak (an effector of Rac and Cdc42). The Rhotekin RBD and Pak PBD can bind to active Rho GTPases and specifically inhibit Rho or Rac/Cdc42-dependent pathways, respectively[18,19]. Expression of the Rhotekin RBD blocked GEF-H1-induced alteration of cell morphology and actin organization, whereas expression of the Pak PBD did not (Fig. 4b). These results suggest that the effects of truncated GEF-H1 constructs on cell morphology rely on the activation of a Rho-dependent pathway. In agreement with this hypothesis, inhibition of Rho-kinase, a downstream effector of Rho, with the pharmacological inhibitor Y-27632 (ref. 20) also prevented the morphological changes induced by GEF-H1. Additionally, expression of a constitutively active RhoA construct, RhoA^{Q63L}, in HeLa cells, promoted changes in cell morphology similar to those induced by truncated GEF-H1 constructs.

Nucleotide exchange activity of GEF-H1 *in vitro*. Our analysis of the morphological effects of GEF-H1 in HeLa cells suggests that mutant versions of GEF-H1 modulate cell shape and actin organization through the activation of Rho. GEF-H1(1–894) was previously reported to be a GEF for both Rho and Rac[10]. To verify that full-length GEF-H1 could promote nucleotide exchange on Rho and to compare the nucleotide exchange activity of various GEF-H1 constructs, we performed *in vitro* measurements of nucleotide exchange on RhoA, Rac1 and Cdc42 in the presence of both full-length and truncated versions of GEF-H1. HA- or EGFP-tagged GEF-H1 proteins were immunoprecipitated from Cos-1 cells and used to catalyse the exchange of GDP for [^{35}S]GTP-γS on Rho GTPases. Both full-length and truncated versions of GEF-H1 promoted nucleotide exchange on RhoA, but not Rac1 or Cdc42 (Fig. 3a). This was in contrast to oncogenic Dbl, which catalysed nucleotide exchange on all three GTPases. As only non-microtubule-localized versions of GEF-H1 induced cell shape changes, we hypothesized that deletion of the N- or C-terminal fragments or mutation of the zinc finger region might enhance the activity of GEF-H1 towards RhoA. However, all GEF-H1 constructs tested (GEF-H1, GEF-H1(1–894), GEF-H1(1–572), KIAA-0651 and GEF-H1^{C53R}) had similar RhoA guanine nucleotide exchange activity (Fig. 3b and data not shown). Thus, deletion of the N- or C-terminal portions of GEF-H1 or mutation of the zinc finger region did not result in significant changes in guanine nucleotide exchange activity *in vitro*.

Activation of Rho family GTPases by GEF-H1 *in vivo*. To address the apparent discrepancy between the fact that all GEF-H1 constructs have similar activity *in vitro* (Fig. 3) and our observation that only non-microtubule-bound GEF-H1 constructs induced a Rho-dependent change in cell morphology (Fig. 2), we set out to analyse the ability of various GEF-H1 versions to activate Rho GTPases *in vivo*. To this end, we first used RBD/PBD pull-down assays[9,21] to measure the amount of active GTPases present in cells expressing various GEF-H1 constructs. With these assays, we detected the activation of RhoA, but not Rac, with various GEF-H1

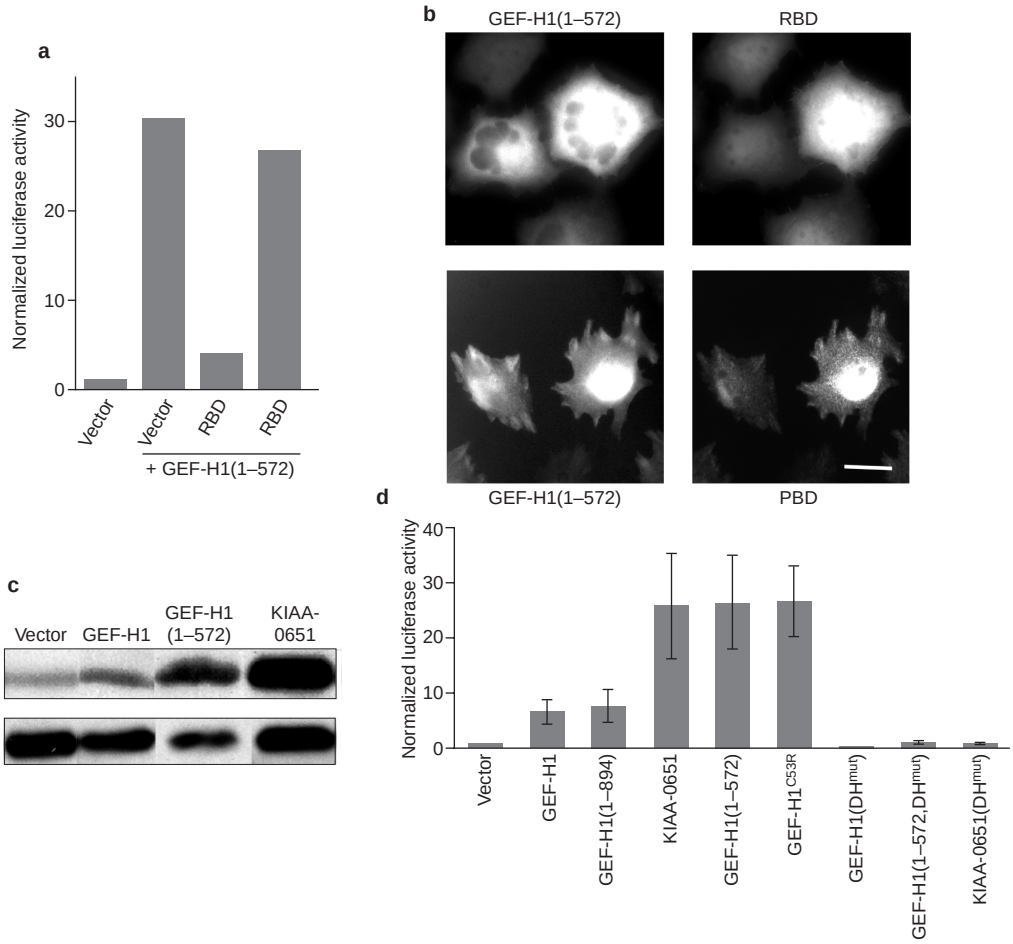

Figure 4 **Activation of Rho by GEF-H1 constructs *in vivo*. a**, Cos-1 cells were transfected with reporter plasmids, GEF-H1(1–572), and RBD or PBD and reporter gene expression was measured (see Methods). The amount of luciferase expressed was divided by the amount of expressed β-galactosidase to adjust for variations in transfection efficiency; vector activity was set to one. SRE activation induced by GEF-H1(1–572) was inhibited by co-expression of RBD. The results shown are representative of at least two independent experiments. **b**, HeLa cells were transfected with HA–GEF-H1(1–572) and EGFP–RBD (top) or EGFP–GEF-H1(1–572) and Myc–PBD (bottom). Cells were stained for GEF-H1 or RBD/PBD, as indicated. The expression of RBD prevented the induction of morphological changes by GEF-H1(1–572), whereas the expression of PBD had no effect. Scale bar represents 20 μm. **c**, HeLa cells cotransfected with GEF-H1 and Myc–RhoA were lysed and GTP-bound RhoA was precipitated with GST–RBD. The amount of RhoA bound to RBD and the level of RhoA expression in whole cell lysates were analysed by western blotting with an anti-Myc antibody. Cells expressing GEF-H1(1–572) or KIAA-0651 contained more GTP-RhoA than cells transfected with full-length GEF-H1. **d**, SRE-luciferase expression induced by the various GEF-H1 constructs was measured as in **a**. Each of the values shown represents the mean (± s.d.) of at least two independent experiments. GEF-H1(1–572), KIAA-0651, and GEF-H1^{C53R} were more active in promoting SRE-luciferase expression than full-length GEF-H1 and GEF-H1(1–894), whereas GEF-H1 constructs with the inactivating mutation (Y393A) in the Dbl domain (GEF-H1(DH^{mut})) showed no activity.

constructs (Fig. 4c and data not shown). Although the RBD assay allowed us to measure RhoA activation, we found that variations in the expression level of Myc–RhoA in those experiments that required co-transfection with additional plasmids (for example, dominant-negative constructs) made routine quantification difficult. To perform more precise measurements of Rho GTPase activation, we used a reporter gene assay that relies on the ability of Rho to activate the transcription of reporter genes fused to the SRE promoter element[22]. Co-expression of GEF-H1 with the SRE-luciferase reporter construct resulted in the upregulation of luciferase expression. This effect was dependent on the nucleotide exchange activity of GEF-H1, as DH domain mutants showed no activity in this assay (Fig. 4d). Interestingly, we observed that non-microtubule-associated GEF-H1 constructs were more active in the SRE reporter assay than full-length GEF-H1 or GEF-H1(1–894) constructs (Fig. 4d). Activation of reporter gene expression induced by GEF-H1 was inhibited by Rhotekin RBD but not by Pak PBD (Fig. 4). Thus, activation of SRE by GEF-H1 seems to be dependent on Rho activity, but not on Rac or Cdc42 activity. These results suggest that the ability of mutant GEF-H1 constructs to induce morphological changes is functionally connected to the higher exchange activity of these proteins, demonstrated by SRE reporter gene activation and the RBD pull-down assay *in vivo*.

GEF-H1 mediates the effects of microtubule depolymerization on Rho activity and cell morphology. Our results indicate that GEF-H1 constructs can be divided into two groups, based on their activity in the SRE reporter assay and their ability to promote changes in cell shape and actin organization — mutant versions of GEF-H1 with highly active guanine nucleotide exchange activity, and the less active full-length and GEF-H1(1–894) proteins. The highly active GEF-H1 constructs are characterized by a lack of microtubule localization, whereas the less active versions bind to microtubules. Thus, it is reasonable to conclude that microtubule association has an inhibitory effect on GEF-H1 activity. Furthermore, the loss of

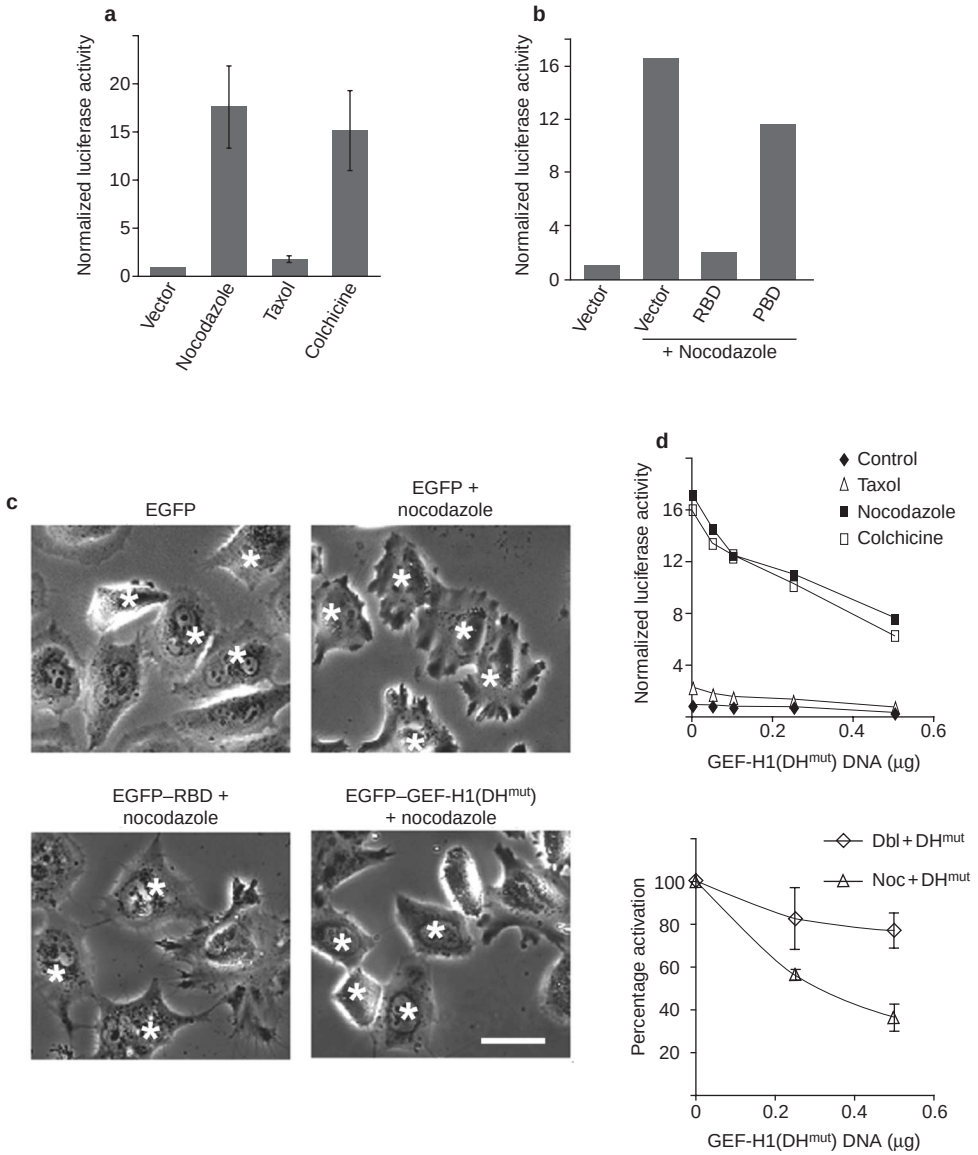

Figure 5 **The effect of microtubule depolymerization on SRE activation and cell shape. a**, Cos-1 cells were transfected with reporter plasmids, treated with microtubule-directed drugs and processed for measurements of luciferase expression (see Methods). Microtubule depolymerization with nocodazole (3.3 μM) or colchicine (2.5 μM) promoted SRE activation, whereas microtubule stabilization with taxol (1 μM) had no effect. Shown are the mean (± s.d.) of six experiments. **b**, Cos-1 cells transfected with RBD or PBD and reporter plasmids were treated with 3.3 μM nocodazole and used to measure luciferase activity. The expression of RBD blocked nocodazole-induced SRE activation. **c**, HeLa cells transfected with EGFP-tagged constructs were imaged by phase-contrast and fluorescence microscopy. EGFP-expressing cells are indicated by asterisks. Treatment with nocodazole (10 μM) resulted in the formation of numerous projections along the cell edge, whereas cells that expressed RBD and GEF-H1(DH^{mut}) maintained a circular shape characteristic of untreated cells. The expression of EGFP alone did not block nocodazole-induced cell shape changes. Scale bar represents 20 μm. **d**, Cos-1 cells transfected with reporter plasmids and varying amounts of GEF-H1(DH^{mut}) DNA were treated with microtubule-directed drugs and luciferase activity was measured (top), as described in **a**. Expression of GEF-H1(DH^{mut}) inhibited SRE activation by microtubule-depolymerizing drugs. Results shown are representative of at least two independent experiments. A comparison of the inhibitory effects of GEF-H1(DH^{mut}) on SRE activation by oncogenic Dbl and nocodazole is shown (bottom). Each data point represents the mean (± s.d.) of three independent experiments.

microtubule localization induced by deletion of the N- or C-terminal amino acid sequences of GEF-H1 or mutation of the zinc finger domain may represent the cause of higher activity of mutant constructs. If this hypothesis is correct, then disruption of microtubules with microtubule-depolymerizing drugs should induce activation of endogenous GEF-H1. Indeed, the microtubule-depolymerizing drugs nocodazole and colchicine promoted Rho-dependent activation of SRE-luciferase reporter gene, whereas the microtubule-stabilizing drug taxol had no effect on SRE reporter gene expression (Fig. 5). Thus, in common with the activation of Rho observed after colchicine treatment of Swiss 3T3 cells[9], disruption of microtubules resulted in the activation of an endogenous, Rho-specific regulatory factor in Cos-1 cells.

Treatment of HeLa cells with nocodazole induced changes in cell morphology and actin organization similar to the changes induced by expression of the highly active GEF-H1 constructs. These morphological changes were blocked by expression of RBD (Fig. 5c). These observations, combined with the results of the SRE

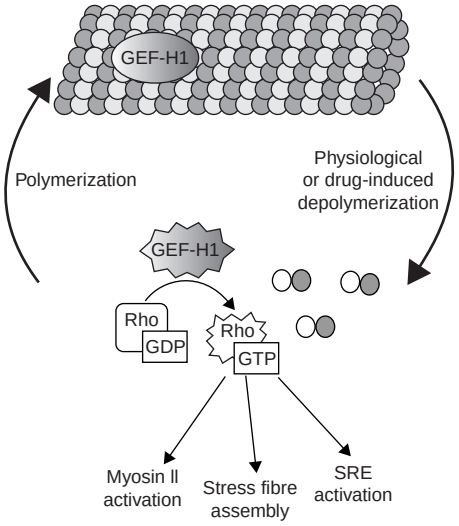

Figure 6 **A model for the regulation of GEF-H1 activity by microtubules.** GEF-H1 is inactive when bound to microtubules and becomes activated when microtubules are depolymerized, either as a result of inherent instability or after treatment with microtubule-depolymerizing drugs. Activated GEF-H1 promotes the binding of GTP to Rho, resulting in the activation of Rho, which in turn induces the upregulation of myosin II contractility, stress fibre assembly and SRE-regulated gene expression.

reporter assay, strongly suggest that microtubule depolymerization activates a Rho-specific nucleotide exchange factor. To verify whether GEF-H1 represents the endogenous factor that is activated by microtubule disassembly, we tested the ability of the DH domain mutant of GEF-H1 to inhibit both SRE activation and the morphological changes induced by microtubule depolymerization. We observed that co-expression of GEF-H1(DH^{mut}) with GEF-H1 partially blocked the effect of GEF-H1 on SRE transcription (data not shown), suggesting that GEF-H1(DH^{mut}) is a dominant-negative inhibitor of GEF-H1 function (see also Methods). Expression of the DH mutant substantially inhibited the SRE activation induced by microtubule disassembly, but had little effect on SRE activation induced by Dbl (Fig. 5), or by the constitutively active heterotrimeric G-protein subunits Gα_{12}QL or Gα_{13}QL, which activate Rho through Rho GEFs that are distinct from GEF-H1 (refs 23,24; data not shown). Expression of the GEF-H1 DH mutant also inhibited nocodazole-induced changes in cell morphology (Fig. 5). The effects of nocodazole were also inhibited by a second dominant-negative GEF-H1 mutant, in which the DH-PH domain tandem had been deleted (see Supplementary Information, Fig. S1). Wild-type GEF-H1 did not block nocodazole-induced SRE activation and cell shape changes, indicating that the inhibitory effects of GEF-H1(DH^{mut}) were not caused by the stabilization of microtubules by GEF-H1. We therefore conclude that GEF-H1, or a closely related Rho nucleotide exchange factor, is responsible for the activation of Rho by microtubule-depolymerizing drugs.

Discussion

In this study, we have analysed the regulation and function of GEF-H1, a microtubule-associated nucleotide exchange factor that is a member of the Dbl family of proteins. We observed that deletion of the N- or C-terminal portions of GEF-H1 resulted in the loss of microtubule localization, suggesting that these regions may be involved in the interaction with microtubules and/or MAPs. However, isolated N- or C-terminal regions did not localize to microtubules (data not shown), indicating that a combination of protein domains may be necessary for microtubule binding. An earlier study reported that the PH domain of Lfc, a mouse homologue of GEF-H1, bound to tubulin[11]. However, we could not detect any interaction between the isolated PH domain of GEF-H1 and tubulin or microtubules. As the previous study did not test the ability of the PH domain to bind to polymerized microtubules[11], it is unclear whether the PH domain of GEF-H1 contributes to microtubule binding. We found that inactivation of the N-terminal zinc finger domain in GEF-H1 by a single amino-acid substitution was sufficient to induce the loss of microtubule localization. Thus, the zinc finger domain may play an important function in the interaction of GEF-H1 with microtubules.

The expression of GEF-H1 constructs that are deficient in microtubule binding induced changes in cell morphology, including cell retraction and the formation of actin stress fibres. This is reminiscent of the changes induced by constitutively active RhoA and suggests that the expression of non-microtubule-associated GEF-H1 results in the activation of RhoA. GEF-H1(1–894) was originally described as a nucleotide exchange factor for Rho and Rac[10]. However, our data indicate that GEF-H1 can promote nucleotide exchange on RhoA, but not Rac or Cdc42 (Fig. 3). Using RBD[9] and PBD[21] pull-down assays to measure the amount of GTP-bound Rho or Rac1, we confirmed that GEF-H1 activated RhoA, but not Rac1, in cells expressing GEF-H1 constructs. These data indicate that GEF-H1 is a nucleotide exchange factor for Rho and are in a good agreement with the observation that Lfc, the mouse homologue of GEF-H1, is also specific for Rho[25]. In support of our conclusions regarding the specificity of GEF-H1 for Rho, our inhibition studies with RBD and PBD indicate that the effects of GEF-H1 on cell morphology and gene expression are mediated by Rho, but not by Rac or Cdc42.

Although all the GEF-H1 constructs had similar guanine nucleotide exchange activity *in vitro*, versions of GEF-H1 that were deficient in microtubule binding were more active in promoting SRE expression and actin reorganization *in vivo*. Thus, we conclude that the loss of microtubule binding induces the activation of GEF-H1. An alternative explanation is that the N- and C-termini of GEF-H1 may function together as an auto-inhibitory module, and that removal of these regions relieves auto-inhibition. However, this seems unlikely, as the truncated and intact GEF-H1 constructs had equivalent activity *in vitro*. Although we observed binding of GEF-H1 to microtubules in detergent-extracted cytoskeletal preparations, we have been unable to reconstitute microtubule binding when microtubules assembled from purified tubulin were added to GEF-H1-containing cell lysates (data not shown). Consequently, we have been unable to demonstrate an inhibitory effect of microtubule binding on GEF-H1 activity *in vitro* (see note added in proof). It is possible that the reconstitution of GEF-H1–microtubule interactions *in vitro* requires some additional components, or that additional conditions (such as GEF-H1 phosphorylation) need to be met. However, in agreement with our hypothesis that microtubule binding (either directly, or through an associated protein component) downregulates GEF-H1 activity, microtubule depolymerization promoted SRE reporter gene expression and changes in cell morphology that were identical to those induced by active GEF-H1. The effects of microtubule depolymerization were inhibited by RBD and GEF-H1(DH^{mut}). Although GEF-H1(DH^{mut}) was able to function in a dominant-negative manner in the signalling pathway activated by microtubule depolymerization, it did not inhibit the SRE activation that is mediated by Dbl or by constitutively active Gα_{12} and Gα_{13}, which are thought to function through other Rho GEFs, such as p115RhoGEF or PDZ RhoGEF[23,24]. By dot-blot assay, we found that GEF-H1(DH^{mut}) bound only weakly to RhoA (see Methods), indicating that it was unlikely to exert its dominant-negative effect simply by sequestering Rho. Thus, the dominant-negative effect of GEF-H1(DH^{mut}) seems to be specific for Rho activation induced by microtubule disassembly and our data strongly indicate that microtubule depolymerization activates

a signalling pathway that involves GEF-H1 and Rho.

The regulation of GEF-H1 activity by microtubule association provides a mechanism for the modulation of Rho activity in response to changes in microtubule dynamics. Microtubule depolymerization can activate Rho by increasing the amount of free, active GEF-H1, whereas microtubule assembly downregulates Rho by sequestering and inactivating GEF-H1 (Fig. 6). This regulatory mechanism may be important in processes that rely on coordinating the activities of the actin and microtubular cytoskeletal systems, such as directional cell migration and cytokinesis. In migrating cells, microtubule depolymerization may locally activate Rho in the cell body, resulting in high myosin II activity and thus promoting tail retraction during locomotion. On the other hand, the prevalence of growing microtubules near the leading edge would result in low Rho activity at the front of the cell, allowing expansion of the leading edge to proceed without being hindered by myosin contractility. The inactivation of GEF-H1 by microtubule polymerization may also provide a molecular basis for the mechanism through which the mitotic spindle defines the position of the acto-myosin cleavage furrow. The furrow is always located between the two spindle poles, and the 'astral inhibition' model suggests that the presence of growing microtubules near the spindle poles locally inactivates myosin and promotes the accumulation of contractile acto-myosin assemblies at the site furthest removed from the microtubule asters[26]. As cytokinesis relies on the activity of Rho[27,28], it seems very likely that the local inhibition of GEF-H1, or a related protein, by astral microtubules may be involved in determining the site of Rho activity and myosin contractility in a dividing cell.

In conclusion, we have identified GEF-H1 as a critical biosensor that links cellular actin polymerization and contractility to changes in microtubule dynamics. These data support a regulatory model in which microtubule dynamics actively regulate cellular signalling mechanisms, inducing the localized activation of Rho GTPases. The contribution of GEF-H1 to other microtubule-dependent signalling events, and alternative mechanisms for regulation of GEF-H1 activity, are currently under investigation.

Note added in proof: We have been able to restore the microtubule localization of the GEF-H1 zinc finger mutant (GEF-H1^{C53R}) by fusing it to the microtubule-binding region of MAP2c. Although this fusion protein exhibits normal guanine nucleotide exchange activity in vitro, *its ability to activate SRE-regulated gene expression* in vivo *is significantly reduced, indicating that forced microtubule binding results in decreased activity of GEF-H1 towards Rho.* □

Methods

DNA constructs

A plasmid containing a cDNA encoding the 894 amino acid GEF-H1 protein was kindly provided by Yong Ren[10]. Our resequencing of this plasmid identified several discrepancies with the originally published sequence. Correction of the sequencing errors resulted in a frame shift that removed the stop codon present in the ORF of the original GEF-H1 sequence. The corrected ORF encoded a protein of 985 amino acids (GenBank accession number: AF486838). Comparison of the full-length GEF-H1 sequence with other protein sequences in the database revealed 88% overall sequence identity with the 985 amino acid mouse protein Lfc (GenBank accession number: 9957220)[11]. This high degree of sequence similarity indicated that Lfc represents the mouse homologue of human GEF-H1. Additionally, a database search identified a protein that probably represents an alternatively spliced form of GEF-H1. This protein is encoded by a human cDNA clone KIAA-0651(ref. 29) and differs from GEF-H1 only in its N-terminus, which does not contain a zinc finger motif (Fig. 1a). We obtained a cDNA clone encoding KIAA-0651 (GenBank accession number: AB014551) from the Kazusa DNA Research Institute (Kisarazu, Japan). All GEF-H1 and KIAA-0651 constructs were subcloned into pCMV5-HA$_3$ or pCMV5-EGFP vectors.

GEF-H1(DH^{mut}) was prepared by site-directed mutagenesis to generate a Tyr to Ala mutation at residue 393 in the conserved QRITKY sequence in the DH domain of GEF-H1. We examined the ability of GEF-H1(DH^{mut}) to bind RhoA with a dot-blot assay. GST-tagged DH-PH-domain protein containing the inactivating DH mutation and recombinant RhoGDI (used as a positive control) were spotted on nitrocellulose and overlayed with RhoA(GDP). Bound RhoA was detected by washing and then immunodetection with a rabbit polyclonal Rho antibody, before binding of ^{125}I Protein A. The DH mutant exhibited only weak RhoA binding that was typically an order of magnitude less than the amount of RhoA bound to RhoGDI on the same blot, over an equivalent range of protein concentrations. The Pak1 PBD (amino acids 67–150) was fused to the Myc-epitope at the N terminus and inserted into pCMV6.

Cell transfection and microscopy

For microscopic observations, HeLa cells were grown on glass coverslips in 35-mm dishes, in DMEM containing 10% foetal calf serum. Cells were transfected with 0.75 μg of each DNA construct and 5 μl of LipofectAMINE reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. At 24 h post-transfection, cells were either examined by phase-contrast microscopy and fluorescence microscopy to detect EGFP fluorescence, or fixed and processed for immunofluorescence staining with anti-HA (UBI, Lake Placid, NY and Covance, Princeton, NJ) or anti-tubulin antibodies (DM1A; Sigma, St Louis, MO), or with Alexa–phalloidin (Molecular Probes, Eugene, OR). Fixation, immunofluorescence staining and imaging were performed as previously described[30]. For some experiments, cells were incubated with 10 μM nocodazole for 1–2 h or 10 μM Y-27632 for 30–60 min.

In vitro exchange assays

HA- or EGFP-tagged GEF-H1 constructs were expressed in Cos-1 cells and immunoprecipitated with anti-HA or anti-GFP monoclonal antibodies and Protein G–Sepharose. Exchange assays were performed with immunoprecipitates, essentially as described[31], except that [^{35}S]GTP-γS was used instead of [^{32}P]GTP. All nucleotide exchange reactions were performed for 15 min at 30 °C.

RBD and PBD pull-down assays

HeLa cells were cotransfected with GEF-H1 constructs and Myc-tagged RhoA or Rac1. After 24 h of expression, cell were then lysed. RBD and PBD assays were performed as previously described[9,21].

SRE reporter gene assay

For transient transfection of Cos-1 cells, 0.5 μg pSRE-luciferase and 0.25 μg pCMV5-LacZ were added per 35-mm dish, as indicator and transfection control plasmids, respectively. GEF-H1 constructs and other plasmids were added at 0.25 μg per well, vector DNA was added to normalize the total DNA amounts. Cells were transfected with LipofectAMINE, in accordance with the manufacturer's instructions, except that the amount of serum was kept at 0.5% throughout the transfection period. Cell lysates were prepared 48 h post-transfection. Luciferase and galactosidase activities were measured with the Luciferase Assay Kit (Promega, Madison, WI) and GalactoLight Kit (Tropix, Bedford, MA). Microtubule-altering drugs were added 12–14 h before lysis at the indicated concentrations (3.3 μM nocodazole; 2.5 μM colchicine; 1 μM taxol).

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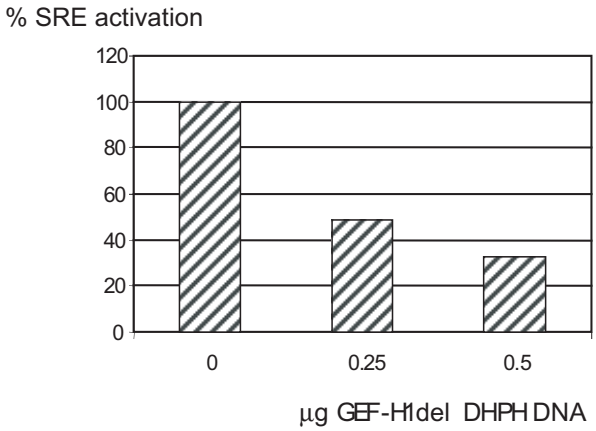

Figure S1 Cos-1 cells were transfected with SRE-luciferase and LacZ reporter plasmids and varying amounts of GEF-H1 construct lacking DH and PH domains (GEF-H1^{delDHPH}). Cells were treated with 3.3 µM nocodazole and expression of reporter constructs was measured as described in Methods. Expression of GEF-H1^{delDHPH} inhibited SRE activation by nocodazole. Results shown are representative of two independent experiments.