

Mechanistic Insight into the Microtubule and Actin Cytoskeleton Coupling through Dynein-Dependent RhoGEF Inhibition

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SUMMARY

Actin-based stress fiber formation is coupled to microtubule depolymerization through the local activation of RhoA. While the RhoGEF Lfc has been implicated in this cytoskeleton coupling process, it has remained elusive how Lfc is recruited to microtubules and how microtubule recruitment moderates Lfc activity. Here, we demonstrate that the dynein light chain protein Tctex-1 is required for localization of Lfc to microtubules. Lfc residues 139-161 interact with Tctex-1 at a site distinct from the cleft that binds dynein intermediate chain. An NMR-based GEF assay revealed that interaction with Tctex-1 represses Lfc nucleotide exchange activity in an indirect manner that requires both polymerized microtubules and phosphorylation of S885 by PKA. We show that inhibition of Lfc by Tctex-1 is dynein dependent. These studies demonstrate a pivotal role of Tctex-1 as a negative regulator of actin filament organization through its control of Lfc in the crosstalk between microtubule and actin cytoskeletons.

INTRODUCTION

The two major components of the cellular cytoskeleton, actin and microtubules, are dynamically coupled to regulate a variety of physiological and pathological cell functions, including polarity, motility, and epithelial barrier permeability (Drubin and Nelson, 1996; Li and Gundersen, 2008; Rodriguez et al., 2003). Microtubule disassembly promotes actin stress fiber formation and enhances cell contraction (Danowski, 1989; Verin et al., 2001). The interplay between microtubules and actin is determined by the control of Rho family GTPases during cycles of microtubule growth and disassembly. Microtubule growth induces Rac activation and promotes lamellipodia formation, while disassembly is associated with activation of Rho and stress fiber formation (Grabham et al., 2003; Liu et al., 1998). The molecular components required for the interplay between microtubule depolymerization and actin stress fiber formation have not been fully elucidated.

Lfc, the murine isoform of ARHGEF2 (also known as GEF-H1 in human) is a microtubule-associated guanine nucleotide exchange factor (GEF) (Ren et al., 1998). An Lfc variant unable to bind to microtubules has increased exchange activity and induces stress fiber formation (Krendel et al., 2002), suggesting that Lfc may be the critical GEF that mediates the crosstalk between microtubules and actin. Subsequent studies have shown that overexpression of Lfc promotes stress fiber formation while its depletion through RNAi attenuates LPA mediated actin reorganization (Birukova et al., 2006; Krendel et al., 2002; Meiri et al., 2009). The molecular components responsible for anchoring Lfc to the microtubule array and the mechanism underlying its inhibited state have yet to be elucidated.

In the present study, we identify Tctex-1 as the factor that couples microtubule depolymerization with actin stress fiber formation. Tctex-1 was originally characterized as a dynein motor light chain (King et al., 1996), although dynein-independent functions of Tctex-1 have been shown to be involved in neuronal growth (Chuang et al., 2005; Sachdev et al., 2007).

Here we show that Tctex-1 maintains Lfc in an inhibited state on microtubules and demonstrate that this function is dynein dependent. These studies support our previous report that Tctex-1 antagonizes Lfc function during cortical neurogenesis (Gauthier-Fisher et al., 2009), which has similarly been shown in n vitro cultured axons (Conde et al., 2010).

RESULTS

Lfc Is Required for Normal Spreading and Attachment of Fibroblasts

To determine the requirement for the endogenous Lfc protein during development and adult life, we created Lfc null mice. These mice were viable with normal life span and no apparent developmental defect, though their litter size and fecundity

Molecular Cell Tctex-1 Couples Microtubules and Actin through Lfc



Figure 1. Altered Morphology, Adhesion, and Actin Stress Fiber Formation in Lfc-Deficient MEFs

(A) Western blot analysis of wild-type MEF and $Lfc^{-/-}$ cell lines.

(B) Representative phase-contrast images of cultured wild-type (MEF) and Lfc-deficient MEFs (Lfc^{-/-}).

(C) Reduced cell surface area of $Lfc^{-/-}$ fibroblasts. Wild-type MEFs, $Lfc^{-/-}$, and $Lfc^{-/-}$ transfected with Lfc-eGFP ($Lfc^{-/-} + Lfc$ GFP) were stained with CellTracker Red CMTPX and DAPI. Cell size was analyzed with Cell Profiler v2.0 (n = number of cells from four independent experiments).

(D) Reduced attachment of $Lfc^{-/-}$ fibroblasts. 300,000 cells were seeded and cultured for 0.5, 2, 6, and 10 hr. At each time point, attached cells were counted following trypsinization. Error bars indicate the standard deviation (SD) of three independent replicates.

were reduced. We derived fibroblast lines from the Lfc^{-/-} and wild-type littermate embryos and verified complete knockout by western blot (Figure 1A). We observed that MEFs lacking Lfc were refractile with elongated extensions compared to wild-type cells (Figure 1B). The average surface area of the Lfc-deficient fibroblasts was reduced by 44% compared to the wild-type cells (p = 0.036), a phenotype that was reversed by expression of Lfc-eGFP (p = 0.066) (Figure 1C). Furthermore, the profound change in morphology observed in the Lfc-deficient MEFs corresponded to a substantial impairment in cell adherence compared to wild-type cells, a defect that was corrected by ectopic expression of Lfc (Figure 1D).

Lfc Induces Actin Stress Fiber and Focal Adhesion Assembly in Mouse Embryo Fibroblasts

Previous reports using RNAi have implicated a role for Lfc in mediating LPA or Thrombin-induced stress fiber formation (Birukova et al., 2006; Meiri et al., 2009); thus we examined actin cytoskeleton remodeling of Lfc-deficient MEFs in response to these stimuli. Wild-type and $Lfc^{-/-}$ cells were stained with phalloidin or anti-vinculin antibodies to visualize polymerized actin and focal adhesions, respectively. Wild-type MEFs rapidly formed bundled actin stress fibers and focal adhesions in response to either LPA or Thrombin, as previously described (Ridley and Hall, 1992). In contrast, Lfc-deficient cells retained cortical actin structures but failed to accumulate stress fibers or focal adhesions (Figures 1E-1G), phenotypes that were reversed by overexpression of Lfc-eGFP (Figures 1E-1G). Further, similar phenotypes were induced in Rat2 cells by Lfc knockdown using three distinct shRNAs (see Figure S1 available online).

One of the Rho effectors required for actin-myosin contraction is ROCK, which phosphorylates myosin light chain (MLC). We observed that Lfc-deficient cells exhibited reduced MLC phosphorylation compared to control cells in response to LPA stimulation, whereas expression of Lfc-eGFP restored MLC phosphorylation (Figures 1H and 1I). Pretreatment of cells overexpressing Lfc-eGFP with the Rho inhibitor C3 blocked induction of stress fibers and focal adhesions (Figure 1J). To examine whether stress fiber induction required the exchange activity of Lfc, we overexpressed a catalytically impaired mutant, Lfc T247F (Figures S5A and S5B) in Lfc^{-/-} cells and found that this mutant was unable to restore stress fiber or focal adhesion formation (Figure 1J). Together, these data demonstrate that Rho activation by Lfc is required for stress formation following LPA treatment in MEFs.

Tctex-1 Binds Directly to the N Terminus of Lfc and Couples It to the Microtubule Array

To identify proteins that may regulate Lfc, we performed a yeast two-hybrid screen using full-length Lfc as bait. We repeatedly isolated a clone that encoded Tctex-1 (Figures S2A and S2B), a dynein light chain (King et al., 1996) that was recently reported to interact with Lfc in cultured axons (Conde et al., 2010).

We constructed a series of His-tagged, N-terminal Lfc deletion mutants (Figure S2C) and found by coimmunoprecipitation experiments that amino acids 87–151 of Lfc are required for Tctex-1 binding (Figure 2A). Using in vitro translated proteins, Flag-Tctex-1 pulled down [³⁵S]methionine-labeled Lfc but not Δ 87-151Lfc, confirming direct binding of Tctex-1 to Lfc residues 87–151 (Figure S2D).

We used bimolecular fluorescence complementation (BiFC) (Hu et al., 2002) to examine the interaction between Lfc and Tctex-1 in vivo. The yellow fluorescent protein Venus was split into N-terminal (VN173) and C-terminal (VC155) fragments, which were fused to Tctex-1 or Lfc, respectively. The Tctex-1:Lfc complex detected by Venus fluorescence exhibited a filamentous pattern (Figure 2B) that colocalized with tubulin fluorescence (correlation coefficient of 0.92, n = 79), indicating that these proteins interact primarily on the microtubule network.

We next examined whether the Tctex-1 binding region of Lfc is required for its microtubule localization. Lfc-eGFP was closely associated with the microtubule array with a correlation coefficient of 0.69 (n = 86), whereas Δ 87-151Lfc-eGFP was distributed almost entirely in the cytoplasm (p = 0.017, n = 67) (Figures 2C and 2F), illustrating the requirement of the Tctex-1 binding region for Lfc's localization to the microtubules. We next examined whether Tctex-1 is required for Lfc localization to microtubules by determining the subcellular localization of Lfc-eGFP in cells from which Tctex-1 had been depleted by RNA interference. Loss of Tctex-1 profoundly altered Lfc localization from a filamentous array to a diffuse cytoplasmic distribution (p = 0.011, n = 61) (Figures 2D-2F). These results demonstrate that Lfc is coupled to the microtubule array by Tctex-1 through an interaction with the Lfc N terminus.

Tctex-1 Regulates the Assembly of Stress Fibers and Focal Adhesions

Given that Tctex-1 binds directly to Lfc on microtubules, we sought to determine if Tctex-1 could modulate Lfc-induced actin cytoskeleton rearrangement in fibroblasts. MEF cells overex-pressing eGFP-Tctex-1 exhibited poorly bundled actin filaments with few stress fibers and significantly fewer focal adhesion

(I) Western blot analysis of Lfc and p-MLC proteins. Lysates from cells treated as in (H) were probed with anti-Lfc, anti-p-MLC, or GAPDH as a loading control. Lower panel: densitometry of the p-MLC bands normalized to GAPDH. The mean intensities of the p-MLC bands are presented, with error bars indicating the standard deviation of three independent replicates.

(J) Cells were starved and treated with 1 µM LPA or C3 prior to the addition of LPA, then fixed and stained with Texas Red phalloidin or anti-Vinculin.

⁽E and F) Confocal images of wild-type MEFs (MEF), Lfc knockout MEFs ($Lfc^{-/-}$), or $Lfc^{-/-}$ MEFs transfected with Lfc-eGFP ($Lfc^{-/-}$ + Lfc GFP). Cells were cultured in growth media (left column) or starvation media for 5 days (second column). Starved cells were treated with 1 μ M LPA (third column) or 3 U/ml Thrombin (fourth column) for 45 min prior to fixation and staining with Texas Red phalloidin to visualize F-actin (E) or anti-Vinculin for focal adhesions (F). Arrows indicate Lfc-eGFP-positive cells. See also Figure S1.

⁽G) Quantification of Vinculin-stained structures per cell representing the average of four independent experiments and total of 225 cells.

⁽H) Confocal images of cells treated as in (E), fixed and stained with Texas Red phalloidin or with an antibody against phosphorylated (S19) myosin light chain (P-MLC).



Figure 2. An Interaction between Tctex-1 and N-Terminal Region of Lfc Is Required for Lfc Localization to Microtubules

(A) Lfc amino acids 87–151 are required for interaction with Tctex-1. HEK293T cells were cotransfected with Flag-Tctex-1 and His-tagged full-length Lfc, Lfc N terminus (1–240), or one of a series of N-terminal Lfc truncations. Tctex-1 immune complexes were immunoblotted with anti-His to detect Lfc and anti-Flag for Tctex-1. The deduced Tctex-1 binding site on Lfc is indicated in the lower panel. See also Figure S2.

(B) Bimolecular fluorescence complementation (BiFC) assays. $Lfc^{-/-}$ cells expressing the N terminus of Venus fused to Tctex-1 (VnTctex-1) and the C terminus of Venus fused to either Lfc (VcLfc) or Δ 151Lfc (Vc Δ 151Lfc) were fixed and stained with anti-Lfc or anti-Tctex-1 antibodies to identify transfected cells and visualized for Venus fluorescence. Cells were also stained with anti- α -tubulin, and colocalization with Venus fluorescence was assessed (bottom right, correlation coefficient of 0.92, n = 79).

(C) Deletion of residues 87–151 of Lfc disrupts microtubule localization. Lfc^{-/-} cells expressing Lfc-eGFP or Δ87-151Lfc-eGFP were fixed and stained for microtubules. Colocalization between GFP (Lfc) and microtubules is shown as an overlay image in the third column.

(D) Tctex-1 knockdown disrupts Lfc localization on microtubules. $Lfc^{-/-}$ cells were cotransfected with Lfc-eGFP and Ds-red-labeled Tctex-1 shRNA or scrambled control, then fixed and stained with anti-Tctex-1. An arrow indicates a cell transfected with the Tctex-1 shRNA (bottom row).

(E) Lfc^{-/-} cells cotransfected with Lfc-eGFP and Tctex-1 shRNA or scrambled control were fixed and stained for microtubules. The third column illustrates the colocalization between Lfc-eGFP and microtubules.

(F) The correlation coefficient measuring colocalization between GFP (Lfc) and microtubules in cells from (C) and (E) in addition to two other independent experiments.

structures in response to LPA or Thrombin treatment (Figures 3A–3C and Figure S3), whereas control cells expressing eGFP alone formed thick, parallel actin stress fibers (Figures 3A and 3B). Rat2 cells stably expressing eGFP-Tctex-1 exhibited similar phenotypes (Figure S3).

Loss of Tctex-1 expression induced profound actin cytoskeletal defects in all cells transfected with the Tctex-1-specific RNAi hairpin. Many cells (35%) exhibited highly disorganized actin fibers, forming branched, interlocking structures composed of poorly bundled filaments in the center of cells (Figures 3D and 3E and Figure S3D). This phenotype was accentuated by LPA or Thrombin treatment (Figure 3D) and was reversed by overexpressing shRNA-resistant Flag-Tctex-1 (Figure S4). Lfc^{-/-} cells transfected with Tctex-1-specific or scrambled shRNA failed to form actin structures following stimulation with LPA (Figure 3D, right panel), indicating that Lfc is required for the Tctex-1 knockdown-induced phenotype.

To test directly the effects of Tctex-1 on Lfc-induced stress fiber formation, we coexpressed full-length Lfc or a truncated form of Lfc (A87-151Lfc) unable to bind to Tctex-1 together with Tctex-1 in Lfc^{-/-} cells. Overexpression of Lfc-eGFP induced formation of parallel actin filaments and focal adhesions (Figure 3F), which were reduced dramatically when Tctex-1 was coexpressed (Figure 3F). Coexpression of Tctex-1 also inhibited Lfc-induced MLC phosphorylation (Figures 3F and 3G). However, ectopically expressed $\Delta 87-151Lfc$ induced stress fiber formation and MLC phosphorylation in a Tctex-1insensitive manner. Moreover, expression of Δ87-151Lfc was consistently more effective than wild-type Lfc in inducing stress fibers, focal adhesions, and p-MLC (Figures 3F and 3G). Together, these data demonstrate that Tctex-1 physically interacts with Lfc and functionally represses Lfc-induced phosphorylation of MLC and formation of actin-based stress fiber structures.

Tctex-1 Inhibits Lfc GEF Activity

We hypothesized that Tctex-1 opposes Lfc function by directly inhibiting its nucleotide exchange (GEF) activity on RhoA. To quantitatively measure nucleotide exchange rates, we employed an NMR-based assay recently developed in our laboratories, which monitors nucleotide-dependent changes in ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra in real time (Figure 4A) (Gasmi-Seabrook et al., 2010; Marshall et al., 2009). In the present study, we monitored the kinetics of RhoA nucleotide exchange catalyzed by full-length GEFs in whole-cell lysates. Remarkably, this approach was sufficiently sensitive to detect the endogenous RhoA-GEF activity in HEK293T cell lysates. Moreover, the precision of this technique enabled us to assay the GEF activities of full-length wild-type and mutant Lfc in whole-cell lysates and determine how their activities are affected by Tctex-1 (Figures S5A and S5B). Lfc-eGFP or Δ87-151Lfc-eGFP were expressed alone or coexpressed with Flag-Tctex-1 in HEK293T cells. We measured the GEF activity of Lfc-eGFP in the lysates using the fluorescence intensity of eGFP (509 nm) to normalize loading. Western blots further confirmed that equal amounts of Lfc or Tctex-1 were used in each assay (Figure 4C). The basal half-life for RhoA nucleotide exchange (GDP to GTP γ S) in the presence of lysate from

eGFP-transfected control cells was approximately 9 hr, whereas expression of Lfc reduced the half-life to 40 min (Figures 4B and 4C and Figures S5A and S5B), but had no effect on Rac nucleotide exchange (Figure S5D). Expression of the catalytically inactive Lfc mutant T247F had a negligible effect on the exchange rate, substantiating the specificity of the assay (Figures S5A and S5B). These data demonstrate that Lfc expression had a potent and specific effect in catalyzing nucleotide exchange on RhoA. Coexpression of Tctex-1 with Lfc-eGFP reduced the exchange activity of Lfc by 80% (Figures 4B and 4C), but did not inhibit PDZRhoGEF (Figure S5C). In contrast, the $\Delta 87$ -151Lfc-eGFP was insensitive to Tctex-1 expression and consistently exhibited 1.6-fold higher GEF activity than Lfc-eGFP, suggesting that amino acids 87-151 encompass a negative regulatory region of Lfc (Figures 4B and 4C).

In light of this finding, we purified recombinant proteins to investigate whether Tctex-1 could inhibit Lfc in a reconstituted system. While recombinant full-length Lfc was catalytically active and bound recombinant rTctex-1 (Figure S5E), it was not inhibited by rTctex-1 in vitro (Figure S5F). In contrast, addition of rTctex-1 to lysates of cells overexpressing Lfc reduced the RhoA nucleotide exchange rate by 50%, confirming the functional integrity of rTctex-1 and suggesting that another cellular factor was required to mediate Lfc inhibition (Figure S5G).

Polymerized Microtubules Are Required for Tctex-1-Mediated Inhibition of Lfc

Because the Lfc:Tctex-1 complex is localized to the microtubules and microtubule-associated Lfc is catalytically inactive, we queried whether the inhibition of Lfc by Tctex-1 was dependent on polymerized microtubules. In the presence of the microtubule depolymerizing agent, nocodazole, wild-type but not $Lfc^{-/-}$ MEFs formed stress fibers; however, expression of LfceGFP reversed this phenotype in the knockout cells, underscoring the requirement for Lfc in mediating the crosstalk between microtubule depolymerization and the induction of actin-based stress fibers (Figure 4D). Whereas coexpression of Tctex-1 with Lfc-eGFP impaired stress fiber formation, treating these cells with nocodazole induced the formation of abundant stress fibers, demonstrating that an intact microtubule array is required for Tctex-1 to suppress Lfc-induced stress fiber formation (Figure 4D).

We used the NMR assay to directly examine how RhoA exchange activity is affected by nocodazole in Lfc-overexpressing cells. The exchange activity in lysates of cells overexpressing Lfc was increased 1.5-fold by nocodazole treatment, consistent with the established inhibitory role of intact microtubules on Lfc activity. Whereas coexpression of Tctex-1 potently inhibited Lfc exchange activity in DMSO-treated cells, no inhibition was observed in nocodazole-treated cells (Figure 4E).

Characterization of a Tripartite Complex between Lfc, Tctex-1, and Dynein Intermediate Chain

These results suggest that Tctex-1 anchors Lfc to microtubules as part of a multiprotein dynein motor complex. We used reciprocal coimmunoprecipitation experiments to examine whether Lfc, Tctex-1, and the dynein intermediate chain (DIC) coexist



Figure 3. Tctex-1 Expression Is Required for Proper Actin Stress Fiber Organization

(A–C) Confocal images of wild-type MEF (first three columns) or Lfc^{-/-} cells (fourth column) expressing either eGFP (first row) or Tctex-1-eGFP (second row). Tctex-1 overexpression suppresses assembly of stress fibers and focal adhesions. Cells were treated with 1 μ M LPA (second column) or 3 U/ml Thrombin (third column), fixed and stained with phalloidin (A) or anti-Vinculin (C). Arrows indicate eGFP-Tctex-1-positive cells. At least 45 images from each condition were counted from three independent experiments. Higher-magnification (5 × 60) views of the boxes depicted in (A) are shown in (B). See also Figures S3A–S3C.

(D) Wild-type MEFs (first three columns) or Lfc^{-/-} (fourth column) cells were transfected with Tctex-1 shRNA or scrambled controls and treated with 1 μ M LPA or 3 U/ml Thrombin, then fixed and stained with phalloidin. Arrows indicate disorganized bundles in the transfected cells. At least 45 images from each condition were counted from three independent experiments.

(E) Higher magnification (5 \times 60) views of the boxes depicted in (D). See also Figures S3D and S4.

(F) Tctex-1 overexpression inhibits induction of stress fibers and MLC phosphorylation by ectopic expression of Lfc but not that of $\Delta 87-151$ Lfc. Lfc^{-/-} cells expressing Lfc-eGFP or $\Delta 87-151$ Lfc-eGFP with or without Flag-Tctex-1 were treated with 1 μ M LPA. Fixed cells were stained with phalloidin, anti-Vinculin, or anti-p-MLC antibodies.

(G) Western blot analysis of Lfc, Tctex-1, or p-MLC proteins in wild-type MEFs, $Lfc^{-/-}$, or $Lfc^{-/-}$ cells expressing Lfc or $\Delta 87$ -151Lfc together with Tctex-1. Densitometries of the p-MLC bands from three independent experiments were normalized to GAPDH.



Figure 4. Tctex-1 Inhibits Lfc GEF Activity in a Microtubule-Dependent Manner

(A) Real-time NMR assay of RhoA nucleotide exchange; a time course of ^{1}H - ^{15}N HSQC spectra acquired during the transition of RhoA-GDP to RhoA-GTP γ S. Red and blue boxes indicate the positions of the cross-peaks for RhoA Q29 in the GDP- and GTP γ S-bound states, respectively. The cross-peak from RhoA E97, which does not undergo significant chemical shift change upon nucleotide exchange, is also indicated. See also Figure S5.

(B) Nucleotide exchange curves in the presence of lysates from HEK293 cells expressing eGFP (black), Lfc-eGFP (red), and Δ 87-151Lfc-eGFP (blue) and coexpressing Lfc-eGFP and Flag-Tctex-1 (orange) or Δ 87-151Lfc-eGFP and Flag-Tctex-1 (green). Each curve is derived from a representative exchange assay, and error bars represent standard deviation of the fraction GDP reported by ten residues.

(C) Nucleotide exchange rates from curves in (B). Standard deviations of three independent replicates are indicated. Lfc and Tctex-1 protein levels in cell lysates were detected in western blots using anti-Lfc and anti-Tctex-1.

as a multiprotein complex in the cell. Endogenous DIC coprecipitated with Lfc-eGFP, and the amount of DIC pulled down was increased with coexpression of Flag-Tctex1 (Figure 5A). When the Tctex-1 mutant T94E, which is unable to bind to DIC (Chuang et al., 2005), was overexpressed, the amount of DIC present in Lfc immune complexes was markedly reduced, consistent with the requirement of Tctex-1 to bridge Lfc and DIC in the complex. Moreover, the Lfc mutant lacking the Tctex1 binding region failed to bring down Tctex-1 and DIC (Figure 5A). Similarly, Lfc was detected in DIC immune complexes and increased with increased Tctex-1 expression (Figure 5A). Immobilized recombinant GST-Tctex-1 fusion protein precipitated both Lfc and DIC from adult mouse brain lysates, confirming that Tctex-1 interacts with endogenous Lfc and DIC proteins. DIC, but not Lfc, was pulled down by GST-RP3, a dynein light chain homologous to Tctex-1, suggesting specificity of Lfc for Tctex-1 in the dynein complex (Figure 5B). Together these results demonstrate that Tctex-1 is required to mediate the association of Lfc with dynein in cells.

To refine the boundaries of the Tctex-1-binding site on Lfc, we purified a recombinant ¹⁵N-labeled fragment comprising the Lfc region mapped in pull-down assays (residues 87–151). ¹H-¹⁵N HSQC spectra collected as ¹⁵N-Lfc₈₇₋₁₅₁ and titrated with Tctex-1 show that resonances assigned to Lfc residues 141–151 became severely broadened (Figures S6A–S6C); thus a construct encompassing Lfc residues 133–161 was generated. Titration of ¹⁵N-Lfc₁₃₃₋₁₆₁ with Tctex-1 caused broadening of resonances assigned to two central regions (143–145 and 148–155) flanked by regions that exhibited chemical shift changes (140–142, 146–147, and 156–160), thus defining residues 139–161 as the Tctex-1 binding site (Figures S6D–S6F).

We next mapped the respective binding interfaces of Lfc and DIC on the Tctex-1 dimer. Titration of ¹⁵N-Tctex-1 with Lfc₁₃₃₋₁₆₁ peptide perturbed resonances assigned (Mok et al., 2001) to both the peripheral α helices and residues near the dimerization interface (Figure 5D and Figure S7B). The pattern of murine ¹⁵N-Tctex-1 resonances perturbed by DIC₁₃₁₋₁₄₃ (Figure S7A) mapped to the edge of the dimerization interface and extend to N40, Q41, R96, and W97 (Figure 5C), consistent with the Drosophila Tctex-1:DIC crystal structure (Williams et al., 2007). Comparison of Tctex-1 resonances perturbed by the Lfc and DIC peptides indicates that Lfc binds to an extensive surface of Tctex-1 (Figures S7C-S7H), which partially overlaps with the DIC binding groove, but extends to a distinct region on the N-terminal α helices (Figure 5). From these NMR titrations, Lfc and DIC peptides were found to bind with similar affinity to Tctex-1, with K_d values of $80 \pm 5 \,\mu$ M and $70 \pm 12 \,\mu$ M, respectively (Figures S7J and S7K). To investigate whether Tctex-1 can simultaneously engage the DIC₁₃₁₋₁₄₃ and Lfc₁₃₃₋₁₆₁ peptides in vitro, we performed a competition experiment. ¹⁵N-Lfc₁₃₃₋₁₆₁ was saturated with Tctex-1, which caused severe peak broadening and some chemical shift changes, and this complex was subsequently titrated with DIC₁₃₁₋₁₄₃. Upon addition of DIC, resonances associated with the unbound state of Lfc residues 144–147 reappeared while other Lfc peaks remained broadened (Figures 5E and 5F). Similarly, when ¹⁵N-Lfc₁₃₃₋₁₆₁ was added to a preformed Tctex-1:DIC complex, all Lfc resonances other than 144–147 became broadened, suggesting that there is an accessible surface on DIC-preloaded Tctex-1 to which Lfc can bind. While the severe peak broadening limits the conclusions that can be drawn from these results, these data strongly suggest that DIC remains associated with Tctex-1 when Lfc binds and is consistent with tripartite interaction between Lfc and DIC with Tctex-1.

Inhibition of Lfc by Tctex-1 Can Be Regulated by Phosphorylation

We next asked whether Tctex-1-mediated inhibition of Lfc is dynein dependent. We examined the capacity of the Tctex-1 phosphomimetic mutant T94E (Chuang et al., 2005) to suppress Lfc activity. The T94E mutation retained interaction with Lfc, albeit with slightly reduced affinity (Figure 6A). However, T94E failed to suppress Lfc exchange activity in the NMR-based GEF assay (Figure 6B), and it failed to inhibit Lfc-induced stress fiber formation in cells (Figure 6C). Importantly, ectopic expression of T94E caused the redistribution of Lfc-eGFP from the microtubule array to the cytoplasm (Figures 6C and 6D). Together, these data demonstrate that the ability of Tctex-1 to inhibit and couple Lfc to the microtubule array is a dynein-associated function.

The Tctex-1-binding sequence on Lfc (139-160) contains S143, recently identified as a phosphorylation site of the polarity kinase Par1b (Yoshimura and Miki, 2011). Phosphorylation of S143 results in the translocation of the Lfc homolog GEF-H1 from the microtubules to the cytoplasm. This suggested to us that S143 phosphorylation may regulate the interaction between Lfc and Tctex-1. We tested the capacity of wild-type, the phosphomimetic S143D, and the nonphosphorylatable S143A point mutant forms of Lfc to coprecipitate with Tctex-1 (Figure 6E). The interaction of Lfc S143D with Tctex-1 was markedly reduced compared to either wild-type or S143A controls. Moreover, Lfc S143D microtubule localization was significantly reduced (Figures 6E–6G), and expression of this mutant strongly induced the formation of parallel actin filaments, suggesting that S143 phosphorylation may be a regulatory switch controlling the localization and activity of Lfc (Figure 6F).

PKA Is Required for Tctex-1-Mediated Inhibition of Lfc

We have previously shown that Lfc is associated with PKA activity and that the PKA agonist Forskolin suppressed the exchange activity of Lfc in cells (Meiri et al., 2009). We queried whether Tctex-1-mediated inhibition of Lfc required PKA

⁽D) Tctex-1 failed to inhibit stress fiber formation in cells treated with nocodazole. Wild-type (MEF) or Lfc^{-/-} cells ectopically expressing eGFP or Lfc-eGFP with or without Flag-Tctex-1 were treated with 1 μ M LPA or with 20 μ M nocodazole, then fixed and stained with phalloidin or anti- α -tubulin antibodies and imaged by confocal microscopy. 120 cells from each of the four conditions were counted from three independent experiments.

⁽E) Nocodazole blocks inhibition of Lfc activity by Tctex-1. Shown are RhoA nucleotide exchange rates in the presence of lysates derived from HEK293T cells overexpressing Lfc-eGFP (red) or Lfc-eGFP and Flag-Tctex-1 (orange), untreated or treated with nocodazole prior to lysis (purple and cyan, respectively). Error bars represent standard deviation of the fraction GDP reported by ten residues.



Figure 5. Lfc, Tctex-1, and DIC Form a Trimolecular Complex

(A) Immunoprecipitation of a tripartite complex of Tctex-1, Lfc, and DIC. LfcGFP and Δ 87-151LfcGFP were overexpressed in HEK293T cells, alone or with wild-type Flag-Tctex-1 or a T94E mutant. Left panel: immunoprecipitation with α -GFP. Right panel: immunoprecipitation with α -DIC. Western blots of the whole-cell lysate (input) and immunoprecipitated complexes (IP) using α -GFP, α -DIC, and α -Flag are shown.

(B) Pull-downs of Lfc and DIC from mouse brain lysate using GST-Tctex-1 and GST-RP3. GST, GST-Tctex-1, and the related dynein light chain GST-RP3 (5 µg) were incubated with lysates prepared from mouse brain. Complexes were precipitated with glutathione-Sepharose beads and resolved by SDS-PAGE (WCL indicates whole-cell lysate).

(C) Tctex-1 residues perturbed by DIC; ribbon model of murine Tctex-1 colored by degree of chemical shift perturbation (shades of red as indicated) upon addition of DIC₁₃₁₋₁₄₃ peptide. Residues that exhibit peak broadening upon DIC addition are colored purple, and those that could not be assessed are gray. Side chains of residues exhibiting chemical shift changes ($[\Delta^{1}H^{2}/(\Delta^{15}N/6.5)^{2}]^{0.5}$) >0.15 ppm are indicated as red sticks. Lower panels: overlaid ¹H-¹⁵N HSQC spectra showing examples of peaks that were highly perturbed by DIC (black, free Tctex-1; red, Tctex-1 plus DIC). Full spectra are shown in Figure S7. (D) Tctex-1 residues perturbed by Lfc₁₃₃₋₁₆₁ peptide (as described in C).

(E) Overlaid ${}^{1}H^{-15}N$ HSQC spectra of ${}^{15}N$ -Lfc(133-161) collected at [Lfc]:[Tctex-1]:[DIC] molar ratios corresponding to 1:0:0 (black), 1:1:0 (red), and 1:1:3 (green). (F) Normalized free peak intensities from ${}^{1}H^{-15}N$ HSQC spectra of ${}^{15}N$ -Lfc(133-161) in complex with Tctex-1 with increasing addition of DIC (molar ratios of [Lfc]:[Tctex-1]:[DIC] were 1:1:1 [blue], 1:1:2 [magenta], and 1:1:3 [green], plotted against Lfc residues 139–161. Note that only resonances from Lfc residues L144–A147 re-emerge in the free position as DIC is added to the Lfc:Tctex-1 complex. phosphorylation. We tested the capacity of Tctex-1 to inhibit Lfcinduced actin polymerization and Lfc GEF activity in cells treated with the PKA selective inhibitor H89. We observed that H89 abrogated Tctex-1 inhibition of Lfc, suggesting that PKA activity is required for Tctex-1-mediated inhibition of Lfc (Figures 6H and 6l). We previously showed that the PKA anchoring protein D-AKAP-1 is associated with Lfc (Meiri et al., 2009). In order to uncouple PKA activity from Lfc, we coexpressed the D-AKAP-1 RII-binding domain peptide with Lfc and Tctex-1 and observed interference with Tctex-1-mediated inhibition of Lfc, confirming the requirement of PKA in this process (Figures 6H and 6l). GEF-H1 has also been shown to be a binding target and substrate for p21-activated kinase 1 (PAK1) (Zenke et al., 2004). We treated cells coexpressing Lfc and Tctex-1 with the PAK selective inhibitor IPA3 (Deacon et al., 2008) and observed no effect on Tctex-1 repression of Lfc, suggesting that PAK1 is not required for this function (Figures 6H and 6I). We previously identified S885 as a major PKA phosphorylation site on Lfc (Meiri et al., 2009). To further investigate the role for PKA phosphorylation in the repression of Lfc by Tctex-1, we coexpressed the Lfc mutant S885A with Tctex-1. We observed that while the S885A mutant bound to Tctex-1 and was normally recruited to microtubules (Figures 6H and 6I), it actively induced stress fibers, and its exchange activity was not inhibited by Tctex-1 (Figures 6H and 6l). These results demonstrate that Tctex-1 anchors Lfc to the microtubules, which facilitates phosphorylation of S885 by PKA, a known Lfc negatively regulatory site, to repress its function.

DISCUSSION

The interplay between the microtubule and actin cytoskeletons is determined, in part, by the coordinated temporal and spatial activation of Rac and RhoGTPases. Pharmacologic disruption of microtubules by nocodazole increases total cellular levels of RhoA-GTP and induces stress fiber formation and cellular contractility, suggesting that polymerized microtubules suppress the activation of RhoA (Bershadsky et al., 1996). The observation that microtubules repress Rho signaling has been attributed to the microtubule sequestration of Lfc in an inactive state (Krendel et al., 2002).

In this study we have identified the dynein light chain, Tctex-1, as the missing link responsible for anchoring Lfc to polymerized microtubules. We previously described a genetic interaction between Lfc and Tctex-1 whereby Tctex-1 antagonizes Lfc function during cortical neurogenesis (Gauthier-Fisher et al., 2009). In the present study we have elucidated the mechanism underlying the epistatic interaction between Lfc and Tctex-1 is an important regulator of the actin cytoskeleton through its capacity to repress Lfc activity.

To determine the mechanism underlying the capacity of Tctex-1 to suppress Lfc function in cells, we devised a quantitative NMR-based RhoGEF assay to measure the catalytic activity of the full-length form of Lfc in cellular lysates. This approach has the advantage of measuring activity of a GEF in the context of its regulatory domains and in the presence of all potential regulatory proteins. We observed that Tctex-1 potently inhibited the exchange activity of full-length Lfc, but not a truncated mutant form of Lfc unable to bind to Tctex-1. Moreover, we noted that the exchange activity of the truncated form of Lfc was higher than the native protein. A transforming variant of GEF-H1 devoid of the N-terminal sequences encompassing the Tctex-1 binding site has been reported in the monocytic leukemia cell line U937 (Brecht et al., 2005). We suggest that this mutation in GEF-H1 might have high constitutive exchange activity due to its failure to be negatively regulated by Tctex-1, explaining its underlying oncogenic potential.

Using NMR spectroscopy, we mapped the Tctex-1 binding sequence of Lfc to residues 139-161, a predicted disordered region lying between the C1 and DH domains. We generated a single point mutation (S143D) in Lfc that disrupted its interaction with Tctex-1 and showed that it had diminished Lfc microtubule localization and potently stimulated stress fibers. Moreover, we determined that Lfc interacts with an extensive surface of Tctex-1 that partially overlaps with the DIC binding site but extends to a region involving the α helices on the lateral aspects of the Tctex-1 dimer. Consistent with our model in which DIC-bound Tctex-1 recruits Lfc to the microtubule array, immunoprecipitation experiments demonstrated that Tctex-1 assembles a tripartite complex with Lfc and DIC in cells. NMR analysis suggests that the three recombinant proteins are sufficient for complex formation in vitro. Although the affinities of Lfc and DIC binding to Tctex-1 are relatively weak, previous work has shown that the dimeric form of DIC has much higher affinity for the dimeric light chains, due to multivalent binding (Hall et al., 2009; Williams et al., 2007). Likewise, full-length Lfc, which contains C-terminal coiled-coil domains, exists as a dimer (D.M. and R.R., unpublished data) and may have higher affinity than the peptide for Tctex-1 in the native state. The partially overlapping binding sites suggest this complex may be mediated by dynamic interactions. We next demonstrated that Tctex-1 T94E mutation, which disrupts DIC binding and microtubule localization, also abolished the ability of Tctex-1 to inhibit Lfc activity. These data show that Lfc inhibition is contingent on its association with a complex including Tctex-1 and the dynein motor intermediate chain.

Using an in vitro reconstituted system, we determined that the Tctex-1:Lfc complex required additional components for inhibition. We showed that intact microtubules are required for Tctex-1-mediated repression of Lfc activity, reinforcing the idea that Lfc must be anchored to microtubules through Tctex-1 in order to be inhibited.

Lastly, we investigated the inhibitory factor associated with polymerized microtubules responsible for Tctex-1-mediated inhibition of Lfc. We previously demonstrated that phosphorylation of S885 by PKA creates a high-affinity 14-3-3 binding site that inhibits the exchange activity of Lfc (Meiri et al., 2009). We have linked this PKA-dependent mechanism of inhibition to Tctex-1, demonstrating that Tctex-1-mediated inhibition involves the PKA anchoring protein, D-AKAP-1 and is dependent on PKA phosphorylation of S885.

We propose a model whereby Lfc is tethered to polymerized microtubules by Tctex-1 and held in an inactive state through PKA phosphorylation. Cycles of microtubule depolymerization would release Lfc from the microtubules (Callow et al., 2005) and alleviate its inhibitory constraints, resulting in a spatially defined activation of Rho (Figure 7). Our data also suggest that



Figure 6. Microtubule Association and PKA Phosphorylation Are Essential for Tctex-1-Mediated Inhibition of Lfc

(A) Tctex-1 T94E retains binding to Lfc. HEK293T cells expressing Flag-Tctex-1 or Tctex-1 T94E together with Lfc-eGFP were lysed, and Lfc protein immune complexes were blotted with either anti-Lfc or anti-Tctex-1 antibodies. Western blot of Lfc and Tctex-1 in the whole-cell lysates are shown (bottom two rows).
(B) Tctex-1 T94E fails to inhibit Lfc activity. Shown are RhoA nucleotide exchange curves in the presence of lysates from HEK293T cells expressing Lfc-eGFP (red), Lfc-eGFP and Flag-Tctex-1 (orange), or Lfc-eGFP and Flag-Tctex-1 T94E (purple). Each curve is derived from a representative exchange assay, and error bars represent standard deviation of the fraction GDP reported by ten residues.

(C) Tctex-1 T94E fails to inhibit Lfc-induced stress fiber formation. Shown are confocal images of cells that were treated with 1 μ M LPA, then fixed and stained with phalloidin or α -tubulin antibodies.



Figure 7. Proposed Model for Inhibition of Lfc by Tctex-1

Lfc is tethered to polymerized microtubules by a Tctex-1 dimer, which interacts with the dynein intermediate chain (DIC) and Lfc at distinct sites to form a tripartite complex. On the dynein complex and the microtubules, Lfc is maintained in its catalytically inactive state through AKAP-dependent phosphorylation of S885 by PKA, which creates an inhibitory 14-3-3 binding site. Microtubule depolymerization releases Lfc from this inhibitory sink, allowing it to stimulate the nucleotide exchange on Rho. Data obtained with phosphomimetic mutants suggest that phosphorylation of Lfc S143 or Tctex-1 T94 would destabilize the interactions between Lfc:Tctex-1 and Tctex-1:DIC, respectively.

phosphorylation of Lfc at S143 or Tctex-1 at T94 may also play a regulatory role in controlling Lfc activation.

Although the RhoGEFs p115RhoGEF, PDZ-RhoGEF, LARG, and AKAP-Lbc have each been implicated in induced stress fiber formation (Sternweis et al., 2007), our data demonstrate that Lfc has a nonredundant and essential role in actin organization. Lfc knockout MEFs failed to form stress fibers and focal adhesion complexes in response to LPA. These cells had reduced surface area and manifested a rounded refractile phenotype with reduced adhesion. This suggests that Lfc's role required for LPA/Thrombin-induced stress fiber formation is determined not only by its level of protein expression and relative GEF activity, but also by its subcellular distribution, mode of regulation, range of GEF specificity against Rho family members, and the multiprotein complex that defines its function.

In this study we have identified the dynein light chain, Tctex-1, as the specificity factor responsible for anchoring Lfc to polymerized microtubules. We have shown that Lfc is indirectly associated with polymerized microtubules and that microtubule-dependent inhibition of Lfc requires the assembly of a multiprotein complex, required to maintain Lfc in its inhibitory state. We have identified a function for Tctex-1 in the production of normal bundled stress fibers. These studies provide mechanistic and structural insight into how the

(D) The correlation coefficient of Lfc-eGFP and polymerized microtubules colocalization in (C) was determined in 60 cells (or more) from three independent experiments.

(E–G) The phospho-mimetic Lfc mutation S143D reduced Lfc Tctex-1 interaction and caused relocalization of Lfc-eGFP from the microtubule array to the cytoplasm. HEK293T cells expressing Lfc-eGFP (wild-type or S143D/A mutants) together with Flag-Tctex-1 were lysed, and Lfc protein complexes were immunoprecipitated using anti-GFP antibody and blotted with anti-Lfc or anti-Tctex-1 antibodies (E). Western blots of Lfc and Tctex-1 in the whole-cell lysates are shown (input, lower panel). Lfc S143D does not localize on microtubules; Lfc^{-/-} cells expressing Lfc S143A-eGFP or Lfc S143D-eGFP were fixed and stained for microtubules and actin (F). The correlation coefficient of Lfc S143A-eGFP or Lfc S143D-eGFP colocalization polymerized on microtubules in (E) was determined in 60 cells from three independent experiments (G).

(H and I) Phosphorylation of Lfc residue S885 by PKA is essential for Tctex-1 mediated inhibition of Lfc. $Lfc^{-/-}$ cells ectopically coexpressing Lfc-eGFP or Lfc S885A-eGFP with Flag-Tctex-1 were starved for 3 days (H). Cells were treated with 30 μ M H89 or 5 μ M IPA3 or cotransfected with D-AKAP-1 RII-binding domain peptide (RII) prior to treatment with 1 μ M LPA, then fixed and stained with phalloidin and imaged by confocal microscopy (H). Shown in (I) are RhoA nucleotide exchange rates in the presence of lysates derived from HEK293T cells overexpressing Lfc-eGFP or Lfc S885A-eGFP and Flag-Tctex-1 or cotransfected with D-AKAP-1 RII-binding domain peptide, untreated or treated with H89 or IPA3 prior to lysis. Standard deviations of three independent replicates are indicated.

microtubule and actin cytoskeletons are coupled in a dyneindependent fashion.

EXPERIMENTAL PROCEDURES

NMR-Based GEF Assay

To measure GEF activity in lysates of mammalian cells, we adapted our recently developed real-time NMR-based assay (Gasmi-Seabrook et al., 2010; Marshall et al., 2009). This assay monitors the heights of ¹H-¹⁵N HSQC peaks of ¹⁵N RhoA protein that are specific to either the GDP-bound or GTP-bound form. To measure nucleotide exchange, 2 mM GTP $\!\gamma S$ and 3.5 µl cleared lysate were added to a 35 µl sample of 0.2 mM ¹⁵N RhoA-GDP (residues 1-181) in NMR buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 2 mM Tris [2-carboxyethyl] phosphine [TCEP], 10% D₂O [pH 7.0]). Nucleotide exchange was monitored by collecting successive ¹H-¹⁵N HSQC spectra at 20°C using 4 or 8 scans (10 or 20 min/spectrum), depending on the reaction rate. Ten pairs of GDP/GTP_γS-specific peaks (R5, V9, Q29, I46, A56, S73, Y74, D87, W158, T163) were used to evaluate the fraction of GDP-bound RhoA present at each time point, and the data were fitted to a single-phase exponential decay function to obtain the exchange rate, as described previously (Gasmi-Seabrook et al., 2010). For more details see Supplemental Experimental Procedures

Other experimental procedures are described in Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/ j.molcel.2012.01.027.

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