Development/Plasticity/Repair

Disregulated RhoGTPases and Actin Cytoskeleton Contribute to the Migration Defect in Lis1-Deficient Neurons

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Lissencephaly is a severe brain malformation caused by impaired neuronal migration. *Lis1*, a causative gene, functions in an evolutionarily conserved nuclear translocation pathway regulating dynein motor and microtubule dynamics. Whereas microtubule contributions to neuronal motility are incompletely understood, the actin cytoskeleton is essential for crawling cell movement of all cell types investigated. *Lis1* haploinsufficiency is shown here to also result in reduced filamentous actin at the leading edge of migrating neurons, associated with upregulation of RhoA and downregulation of Rac1 and Cdc42 activity. Disruption of RhoA function through pharmacological inhibition of its effector kinase, p160ROCK, restores normal Rac1 and Cdc42 activity and rescues the motility defect in *Lis1*+/− neurons. These data indicate a previously unrecognized role for Lis1 protein in neuronal motility by promoting actin polymerization through the regulation of Rho GTPase activity. This effect of Lis1 on GTPases does not appear to occur through direct Lis1 binding of Rho, but could involve Lis1 effects on Rho modulatory proteins or on microtubule dynamics.

Key words: **Lis1; platelet activating factor acetyl hydrolase 1b1 (Pafah1b1); neuronal migration; cerebellar granule neurons; RhoA; Rac1; Cdc42**

Introduction

Neuronal migration is essential to brain formation, allowing neurons born in the germinal ventricular zone to reach their final positions (for review, see Hatten, 1999). Studies of one gene associated with cortical malformation, *Lis1*, have been especially fruitful in identifying molecular mechanisms of neuronal motility (Vallee et al., 2000). *Lis1* mutations cause Miller-Dieker lissencephaly, and the gene encodes a noncatalytic subunit of the enzyme platelet-activating factor acetylhydrolase, Pafah1b1 (Reiner et al., 1993; Hattori et al., 1994; Ho et al., 1997). Pafah1b1, hereafter Lis1, is highly conserved and seems to have similar functions in fungus, *Drosophila*, and mouse (for review, see Morris et al., 1998a; Feng and Walsh, 2001; Ross and Walsh, 2001; Wynshaw-Boris and Gambello, 2001). Lis1 belongs to the WD40 superfamily known for multiple protein–protein interactions (Garcia-Higuera et al., 1996). Lis1 interacts with cytoplasmic dynein and proteins of the microtubule organizing center (MTOC), including nuclear distribution-E (NudE) in fungus and its mammalian homologs mNudE and NUDEL (NudE-like) (Feng et al., 2000a; Kitagawa et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000; Sweeney et al., 2001). Lis1 deficiency in all species examined produces nuclear translocation defects, abnormal positioning of the MTOC, and altered distribution of the Golgi apparatus, consistent with lost cytoplasmic dynein function (Faulkner et al., 2000; Liu et al., 2000; Smith et al., 2000).

Whereas complete loss of Lis1 produces embryonic lethality, *Lis1* haploinsufficiency disrupts neuronal migration (Hirotsune et al., 1998). Altered motility of *Lis1*+/− neurons may result from defective nuclear translocation through impaired interactions with NudE homologs, cytoplasmic dynein, and effects on the MTOC. A related hypothesis links Lis1 and cyclin-dependent kinase 5 (Cdk5), which phosphorylates a Lis1 interacting protein, NUDEL (Niethammer et al., 2000). This is supported by evidence that loss of Cdk5, a serine–threonine protein kinase, causes defective neuronal migration in mice (Ohshima et al., 1999). The microtubule cytoskeleton might also be affected in *Lis1*+/− neurons. Intact microtubules are required for axon elongation, and Lis1 has been shown to stabilize microtubules *in vitro* (Sapir et al., 1997). However, in fungus, Lis1 promotes microtubule dynamics (Han et al., 2001). Therefore, the *in vivo* role of Lis1 in regulating neuronal microtubules remains to be clarified.

In the course of examining cultured LIS1-deficient neurons by videomicroscopy, we observed several motile abnormalities. Because an intact actin cytoskeleton is essential for neuronal motility (Rivas and Hatten, 1995), the present study examined whether *Lis1* haploinsufficiency could also disrupt neuronal migration through effects on the actin cytoskeleton. Consistent with a role for Lis1 in regulating actin-based motility, *Lis1*+/− neurons displayed a markedly reduced filamentous actin (F-actin) content at the leading edge, as well as fewer and shorter filopodia.

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Polymerization of actin drives the protrusion of the leading edge, and Rho GTPases are key regulators of F-actin in response to extracellular stimuli (Bishop and Hall, 2000). The GTPases Rac1 and Cdc42 promote polymerization at the leading edge, whereas RhoA antagonizes this effect, promoting retraction of the leading edge and the assembly of stress fibers (Schmitz et al., 2000). Therefore, we investigated whether *Lis1+/−* neurons have abnormal activity of the Rho family of small GTPases. The data support the hypothesis that Lis1 has a previously unrecognized additional role in neuronal motility in which Lis1 promotes actin polymerization through signaling that inhibits RhoA. Based on existing studies, this could reflect indirect interaction between Lis1 and GTPases either through GTPase modulatory proteins or through microtubules.

Materials and Methods

Cell culture and pharmacological agents. Cerebella from early postnatal mice (P3–P7) were dissociated using established procedures (Gasser and Hatten, 1990). For studies of glia-guided migration, plating procedures were used as described previously (Gasser and Hatten, 1990). In neurite-guided migration assays, dissociated cells were preplated on bacteriological Petri dishes overnight. This resulted in the formation of neuronal aggregates that were loosely attached to the plate and were collected and replated onto laminin-coated slides (25 μg/ml). Granule cell reaggregates were maintained in BMEM (Invitrogen, Gaithersburg, MD) containing 10% horse serum, 10% FBS, 100 U each of penicillin and streptomycin, 200 μM glutamine, and 6 mM glucose. Cells were cultured at 37°C in 5% CO_2. After 24 hr in culture, cells were fixed in 0.5% glutaraldehyde. Alternatively, cultures were incubated for another 6 hr in the presence of Rho-kinase inhibitor, 10 μM HA-1077 or 10 μM Y27632, singly or together (Biomol, Plymouth Meeting, PA) or with the drug vehicle, and then either fixed for histological analysis or cell lysates were collected for assessment of GTPase activity by Western analysis (see below). For assessment of direct Rho inhibition, 24 hr after plating, cells were loaded with C3 transferase (Cytoskeleton Inc., Denver, CO), using the Pro-Ject protein loading reagent (Pierce, Rockford, IL) in serum-free media as directed. After a 3 hr incubation in C3, cells were lysed, and Rac1 and Cdc42 activity was analyzed using a small GTPase assay (Pierce) according to the manufacturer's protocol. Neuronal purity of cultures was assessed by dual staining of neurons with anti-βIII-tubulin (Tuj1; Covance, Princeton, NJ) and glia with anti-GFAP antibodies (Sigma, St. Louis, MO).

Fibroblasts for motility assays were obtained from cerebella after the preplating step. The strongly adherent cells left on the plastic after loosely attached neurons had been removed were trypsinized, replated onto poly-D-lysine-coated Petri dishes, and grown to confluence. After several passages, these cultures consisted of only fibroblasts as judged by the characteristic cell morphology and the absence of neuron-specific, βIII-tubulin, and glia-specific GFAP antigens in the cell lysates, when analyzed by Western blotting.

Videomicroscopy for dynamic assessment of cell motility. Granule cell or fibroblast migration was visualized by phase-contrast video microscopy of live cultures. The temperature on the microscope stage was maintained at +37°C using an air-stream incubator. During recording, cells were kept in L-15 (Invitrogen) supplemented with 8 mM glucose. Each recording session lasted 1.5–3 hr, and one frame was taken every 3 min. Independent of the substratum, neurons moved by extending a short leading process rapidly followed by pulling up of the cell body. Therefore, cell movement was measured by the displacement of the center of the cell (centroid) calculated using MetaMorph (Universal Imaging, Downingtown, PA) software between the frames. Because the granule cell body is made up primarily of nucleus with the surrounding cytoplasm comprising only ~10–20% of the volume, this measurement is comparable with the one made for fibroblasts from the center of the nucleus. In all fibroblasts observed, the cell nucleus could be reliably identified because of its flattened morphology. Only centroid or nuclear displacements of 1 μm and larger over 3 min intervals between two consecutive frames were considered.

Migration analysis by cell distribution in reaggregates at 24 hr in culture. Two criteria were used to select aggregates of cerebellar granule neurons for analysis. First, variability in the number of cells per aggregate was limited by using only those clusters with a diameter between 90 and 150 μm. Second, only those aggregates were analyzed whose axonal fascicles did not contact neurites or cells from another aggregate. The effects of pharmacological treatments and genotype on neuronal migration were analyzed by the distribution of cells migrating from the aggregates along the axonal fascicles. Every fascicle was divided into 50 μm segments. The number of migrating granule neurons was counted for every segment.

Immunocytochemistry and quantification of fluorescence intensity. Glass slides were coated consecutively with 50 μg/ml poly-D-lysine and 25 μg/ml laminin for 3 hr at 37°C each. Dissociated wild-type (wt) and Lis1+/− granule neurons were plated on the slides and cultured for 24 hr. Cells were then fixed in 0.2% glutaraldehyde and briefly permeabilized with 0.1% Triton X-100. The slides were blocked in 10% goat serum before incubation with antibodies. Cells were treated with anti-βIII-tubulin (Tuj1) antibody (Covance), followed by a rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) and fluorescein-labeled goat anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA). Cell fluorescence was examined on a compound microscope (model AX-70; Olympus, Lake Success, NY) connected to a laser confocal scanning head (model 1024; Bio-Rad, Hercules, CA). Quantitative analysis of fluorescence intensity and three-dimensional reconstruction of image data were performed with MetaMorph software (Universal Imaging).

Western blotting and Rac1, Cdc42, and RhoA activation assay. The determinations of the active GTP-bound forms of Rac1, Cdc42, and RhoA were performed with a pull-down assay, according to the manufacturer's protocol. PAK1-PBD or Rhotekin-RBD pull-downs were analyzed by Western blotting with Rac1 antibody (Upstate Biotechnology, Lake Placid, NY), Cdc42 antibody (Cytoskeleton), or RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Films were scanned on an optical densitometer (Bio-Rad), and relative protein concentrations were determined using Molecular Analyst software (Bio-Rad) by comparing the optical densities of the specific bands of appropriate apparent molecular weight. Optical densities were normalized to the total amount (GTP bound and unbound) of the appropriate GTPase in each lysate, determined from Western blot analysis of electrophoretic gels loaded with equal amounts of protein from the lysates.

Statistical analysis. For the measurements of fluorescence intensity, cell migration and time-lapse video microscopy of at least three independent experiments were performed with a minimum of seven observations for each treatment in every experiment. A two-tailed Student's *t* test was used to determine the significance of the difference between the means. For the optical densitometry of Western blots, at least three independent experiments were performed with lysates derived from littermates of three or more different litters. The significance of the mean variation was determined using the nonparametric Mann–Whitney *U* test.

Results

Deficient migration of *Lis1+/−* cerebellar granule neurons on glial or neuritic fibers

Because the major mode of neuron movement in cortical regions is along radially oriented glial fibers, we first examined Lis1-deficient neurons moving on glia (Fig. 1*A–E*). Glia-guided migration was confirmed by dual immunolabeling of cultures using anti-βIII-tubulin for neurons and anti-GFAP for glia (Fig. 1*B*). By time-lapse video microscopy, *Lis1+/−* cells exhibited a 50% reduction in average velocity compared with wild type (Fig. 1*C*). This was associated with a reduced maximal velocity and increased time spent at rest (Fig. 1*D*, *E*)

In certain locations, neuronal motility in brain also occurs along neuronal fibers (Wichterle et al., 1997; Komuro and Rakic, 1998). A reaggregate assay of granule cells on neurites enables observation of more neurons undergoing vectorial movement (Fig. 1*F–J*). Double immunostaining with anti-βIII-tubulin and

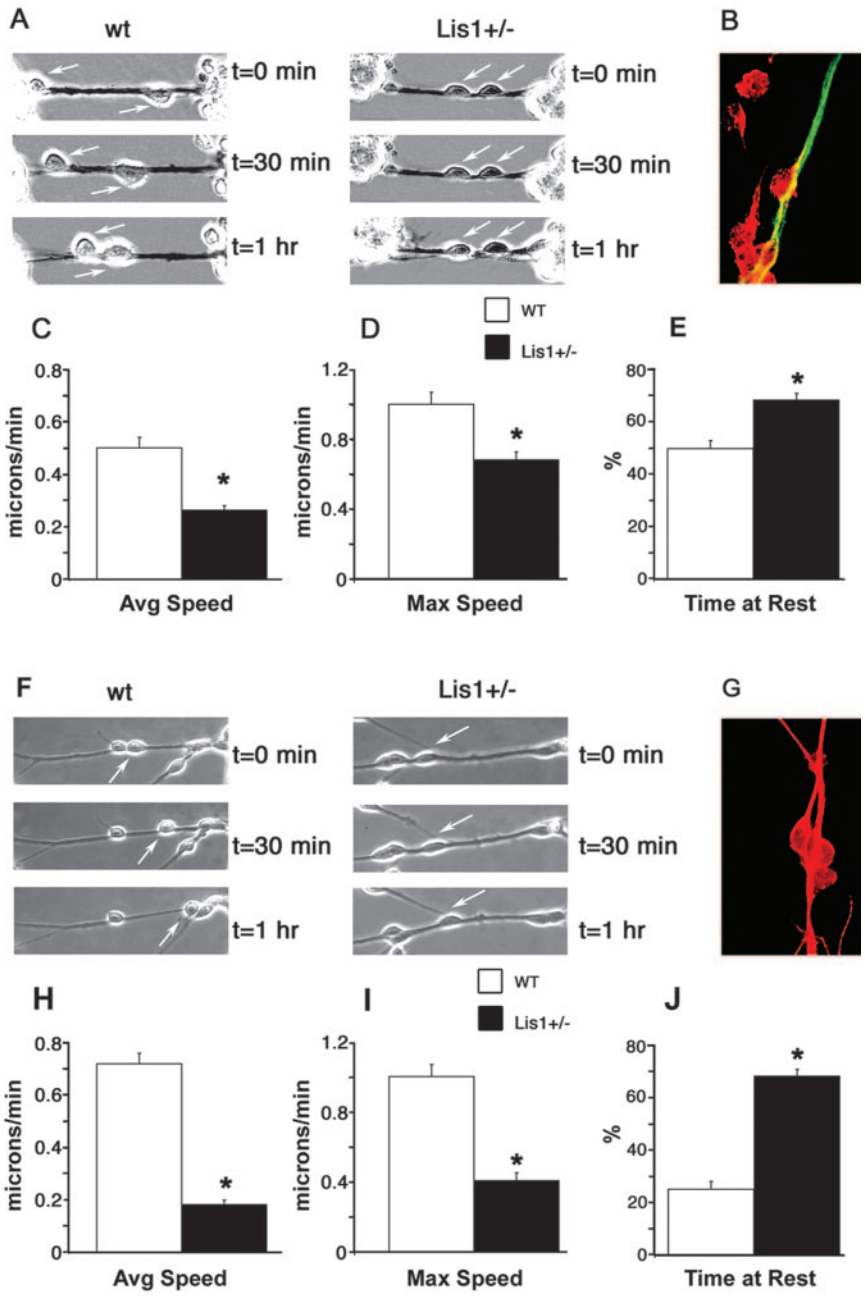

Figure 1. Lis1+/− cerebellar granule neurons display motility defects in both glia- and neurite-guided migration assays. *A*, Wild-type neurons cover a longer distance when migrating on glia than Lis1+/− neurons, as shown by displacement of the cell body (arrow) over the period of 1 hr (video-enhanced phase–contrast microscopy, 30 min between planes). *B*, Glia-guided assay cultures stained with βIII-tubulin (neurons; red) and GFAP (glia; green) antibodies. *C*, Average speed of Lis1+/− neurons ($n = 17$) compared with wt neurons ($n = 43$). *D*, The maximal speed of neurons was decreased in Lis1+/− neurons, whereas the percentage of time at rest (*E*) was increased. *F*, In reaggregate cultures, neurons cover a longer distance when migrating on neurites than Lis1+/−, neurons as shown by displacement of the cell body (arrow) over the period of 1 hr (video-enhanced phase–contrast microscopy, 30 min between planes). *G*, Dual labeling with βIII-tubulin (neurons; red) and GFAP (glia; green) antibodies show reaggregate cultures are >95% neurons. Average speed (*H*), maximal velocity (*I*), and time spent at rest (*J*) are all negatively affected in Lis1+/− neurons on neurites ($n = 12$) compared with wt neurons ($n = 21$). *$p < 0.01$.

anti-GFAP revealed that >95% of cells in the reaggregate cultures were neurons (Fig. 1*G*). Migration along neurite fascicles extended from the aggregates was recorded by high-resolution time-lapse video microscopy (Fig. 1*F*,*H–J*). As in classical studies of glia-guided neuronal movement, this migration was saltatory, with periods of movement interspersed with rest (Edmondson and Hatten, 1987). There was a nearly fourfold reduction in the average speed of migration of *Lis1*+/− neurons along neurite fascicles, compared with wt littermates (Fig. 1*H*). The deficient migration of *Lis1*+/− cells was characterized by both a reduced maximal velocity and prolonged periods at rest (Fig. 1*I*,*J*).

Having validated the migration defect by time-lapse video microscopy of granule cells on glia as well as neurites, additional experiments examined whether a migration defect could be detected in reaggregate cultures by a simpler assay involving assessment of single frames taken at 24 hr in culture. Figure 2 compares the distribution of granule cells that had migrated along the neurite fascicles that were extended from clusters of *Lis1*+/+ granule neurons (Fig. 2*A*), compared with similar sized clusters of *Lis1*+/− cells (Fig. 2*B*). By 24 hr, significantly fewer cells had migrated from the *Lis1*+/− reaggregates at all distances measured along the neurite fascicles, and an 88% reduction ($p < 0.002$) in the number of neurons was found at a distance of 150 μm from an aggregate cluster (Fig. 2*C*). These time-lapse and single-frame experiments demonstrated that the migration deficit could be detected by determining the distribution of cells in the reaggregate assay at 24 hr and that this measure was relevant to neuronal movement. Therefore, this bin assay, which was conducted more rapidly, provided a reasonable representation of neuronal motility.

This reaggregate assay also demonstrated a defect in axon extension by *Lis1*+/− granule cells after 24 hr in culture (Fig. 2*D*). The mean fascicle length was 175 μm in *Lis1*+/− clusters, compared with 210 μm in wt cells, a difference of 15% ($p < 0.0001$). However, the number of neurite fascicles per cluster did not differ significantly.

Lis1+/− neurons display abnormalities of the actin cytoskeleton

The time-lapse video microscopy studies revealed altered leading processes and filopodia in Lis1-deficient neurons, suggesting abnormalities in the actin-based cytoskeleton. Therefore, we sought to determine whether alterations in F-actin could be associated with *Lis1* haploinsufficiency. Cultured cerebellar granule neurons were immunostained with βIII-tubulin antibody, whereas F-actin was visualized with rhodamine–phalloidin. The cytoskeletal organization of isolated, migrating neurons is similar to those moving on glia (Rivas and Hatten, 1995). Therefore, measurements were made on neurons isolated on the coverslip, to permit reliable quantification of fluorescence. Granule neurons were selected for analysis on the basis of migratory morphology, as de-

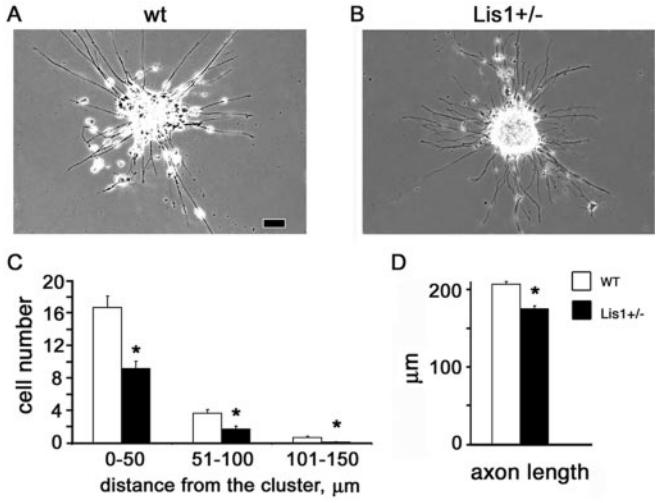

Figure 2. Fewer Lis1+/− cerebellar granule neurons migrate out of reaggregate clusters, and axonal fascicles are shortened. After 24 hr on laminin, wt neurons (*A*) actively move out from the reaggregate clusters and start migrating along neurite fascicles. Far fewer cells migrated from clusters of *Lis1*+/− neurons (*B*). *C*, Cell numbers in every 50 μm radial bin are significantly reduced in *Lis1*+/− ($n = 60$ clusters) compared with wild type ($n = 60$). *D*, Axons extending from clusters of Lis1+/− neurons ($n = 826$ neurite fascicles) are significantly shorter compared with wild type ($n = 847$ neurite fascicles). Scale bar, 50 μm. *$p < 0.01$.

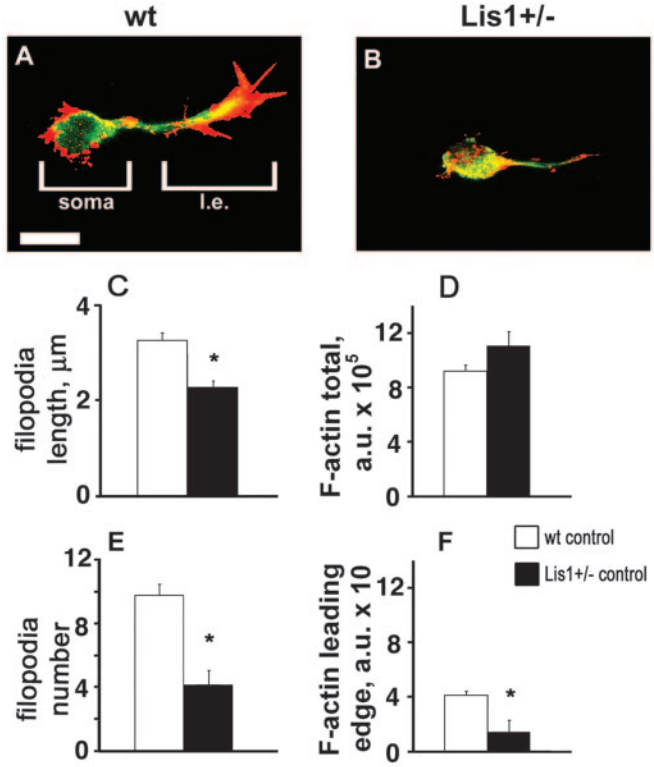

Figure 3. F-actin is reduced at the leading edge and increased in the soma of Lis1+/− neurons, whereas the number and length of filopodia are reduced. Cultured cerebellar granule neurons stained with rhodamine–phalloidin (red) and βIII-tubulin (green). *A*, Wild-type neurons have a characteristic leading edge (l.e.) containing several filopodia and a cell body (soma) with little F-actin. *B*, Lis1+/− neurons have a leading edge dramatically reduced in size, fewer filopodia, and an increased amount of F-actin in soma (yellow overlap of labels). Filopodia length (*C*) and number (*E*) are reduced in Lis1+/− cells ($n = 33$) compared with wt cells ($n = 41$). Whereas the total amount of F-actin labeling was unchanged (*D*), the amount of F-actin at the leading edge (*F*) was reduced in Lis1+/− cells. Scale bar, 10 μm. a.u., Arbitrary units. *$p < 0.01$.

scribed by Rivas and Hatten (1995). The diameter of the soma had to be <12 μm with scant cytoplasm, and the length of the leading neurite had to be <40 μm, consistent with a leading process rather than a growth cone.

Lis1 has been implicated as a regulator of microtubules. Therefore, we first sought to evaluate the microtubule component of the cytoskeleton. Staining of neurons with βIII-tubulin antibody did not reveal obvious abnormalities in the organization of the microtubule cytoskeleton in Lis1+/− neurons (Fig. 3*A*,*B*). The distribution of Tuj1 staining in Lis1-deficient granule neurons appeared indistinguishable from wt cells, and the fluorescence intensity was preserved (wt, 41 cells: $6.6 \times 10^5 \pm 0.6 \times 10^5$; Lis1+/−, 33 cells: $6.2 \times 10^5 \pm 0.6 \times 10^5$).

Phalloidin-labeled F-actin was visibly reduced at the leading edge in *Lis1*+/− neurons (Fig. 3*A*,*B*). The amount of F-actin present in *Lis1*+/+ and *Lis1*+/− neurons was estimated by summation of the phalloidin labeling in optical sections recorded along the *z*-axis of the entire volume of either the reconstructed neuron or its leading process. Compared with wild type, F-actin in *Lis1*+/− neurons was significantly reduced in the leading process (34% of control; $p < 0.001$), whereas the total amount of phalloidin staining across the entire cell was unchanged (Fig. 3*D*,*F*). In *Lis1*+/− neurons, the decrease in phalloidin staining in the leading process was accompanied by an increase in fluorescence intensity in the cell body (note the increased yellow overlap of tubulin and actin in the Lis1-deficient cell body in Fig. 3*B*), reduced length, and a reduced number of filopodia (Fig. 3*C*,*E*).

Lis1+/− cerebellum contains reduced levels of activated Rac1 and Cdc42 and increased level of activated RhoA

Additional experiments investigated the activities of Rho GTPases, which are known to regulate the actin cytoskeleton (Schmidt and Hall, 1998). Rac1 and Cdc42 have been shown to promote actin polymerization at the leading edge of motile cells, whereas RhoA antagonized this activity, promoting retraction of the leading edge and assembly of stress fibers (Schmitz et al., 2000). Therefore, the activities of these Rho GTPases were compared in *Lis1*+/− and *Lis1*+/+ neurons. As summarized in Figure 4, Lis1-deficient cerebellar tissue contained levels of activated Rac1 that were 43% (43.06 ± 3.67%) of wt controls and levels of Cdc42 that were 18% (17.52 ± 4.74%) of wt cells (Fig. 4*A*,*B*,*D*). Thus, *Lis1* haploinsufficiency was associated with a large downregulation of the activated forms of Rac1 and Cdc42, as measured by pull-down assays with PAK1-PBD that specifically binds activated forms of Rac1 and Cdc42. Consistent with the role of RhoA as an antagonist of Rac1/Cdc42 activity, GTP-bound RhoA was elevated in *Lis1*+/− cerebella more than three times that found in wt neurons (345.4 ± 28.26%; Fig. 4*C*,*D*).

Inhibitors of the RhoA effector kinase p160ROCK upregulate activated Rac1/Cdc42 and rescue defects in the actin cytoskeleton, migration, and axon elongation *in vitro*

The observation that active Rac1 and Cdc42 were severely reduced in *Lis1*+/− neurons prompted investigation of whether RhoA influences Rac1 and Cdc42 activity in granule neurons in a manner similar to other systems. The RhoA-associated kinase p160ROCK has been the best characterized downstream effector of RhoA (Bishop and Hall, 2000). Thus, pharmacological p160ROCK inhibitors HA1077 and Y27632 were used to examine whether RhoA activity regulated levels of activated Rac1/Cdc42

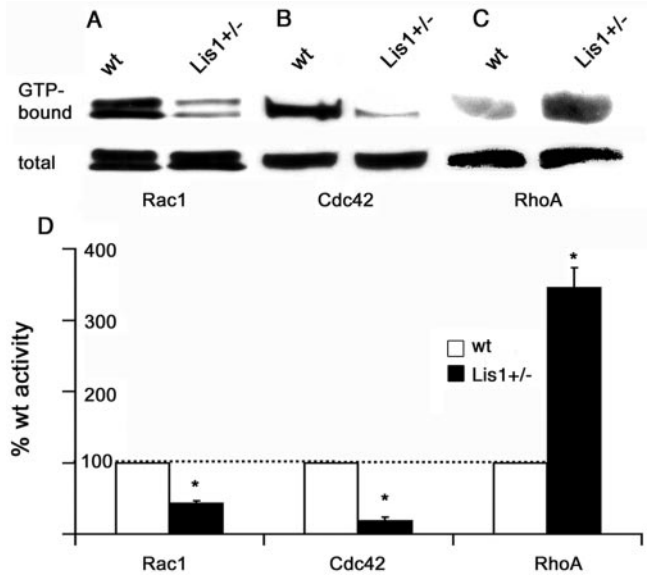

Figure 4. Reduced levels of active Rac1, Cdc42, and increased active RhoA in *Lis1*+/− cerebellum. *A–C*, Pull-down assays reveal decreased GTP-bound Rac1 (*A*) and Cdc42 (*B*), whereas GTP-bound RhoA is increased in brain lysates of Lis1+/− animals. Total levels of Rac1, Cdc42, and RhoA proteins are unchanged. *D*, Activity of Rho GTPases expressed as percentage of control level is reduced in Lis1+/− cells ($n = 3$ experiments; 12 animals per genotype). *$p < 0.01$.

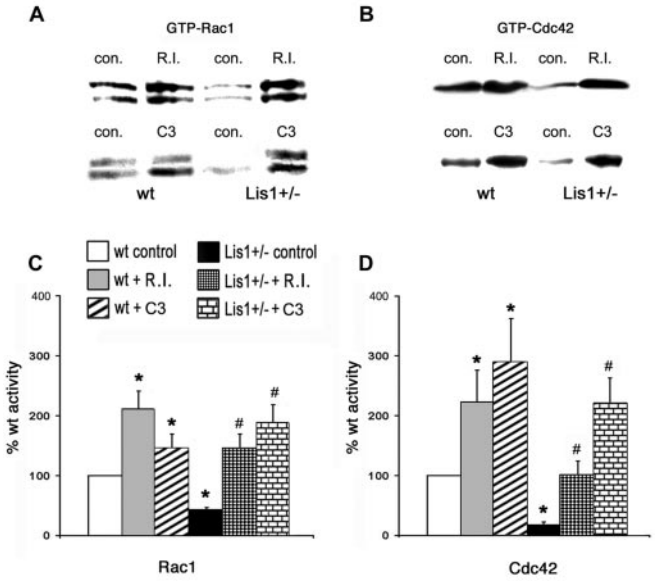

Figure 5. p160ROCK inhibitors or C3 restore normal levels of active Rac1 and Cdc42 in *Lis1*+/− neurons. *A*, *B*, Treatment of either wt or Lis1+/− neurons with either p160ROCK inhibitors (R.I.) or RhoA inhibitor (C3) upregulates the amount of GTP-bound Rac1 (*A*) and Cdc42 (*B*). Treatment of wt neurons with p160ROCK inhibitors increases levels of active Rac1 (*C*) and Cdc42 (*D*) twofold and restores wt levels of these GTPases in Lis1+/− neurons ($n = 3$ experiments; 4 cultures of each genotype per condition or 16 cultures per experiment). Treatment of cultures with C3 transferase, a direct inhibitor of RhoA, produces a similar upregulation of Rac1 and Cdc42 (*C*, *D*). *$p < 0.01$ compared with untreated wt control; #$p < 0.01$ compared with untreated Lis1+/− control.

in these *Lis1*+/− neurons (Fig. 5). Treatment of granule cell cultures with either or both HA1077 andY27632 restored activity of Rac1 and Cdc42 in *Lis1*+/− neurons to wt levels and enhanced these activities in wt neurons (Fig. 5*C*,*D*). The response to the two drugs together was slightly more robust with less variability among experiments, and so the combination of compounds was used in the data presented. Whereas the levels of activated Rac1 and Cdc42 in wt cerebellar neurons were doubled by ROCK inhibitors, these activated GTPases in Lis1-deficient neurons quadrupled, with Rac1 rising above wt untreated levels, although not quite as high as wt neurons treated with ROCK inhibitors (Fig. 5*C*,*D*). To further validate the involvement of Rho A in this activity, separate cultures were treated with C3 transferase, a potent direct inhibitor of Rho (Williamson et al., 1990). Administration of C3 had similar biochemical effects to the p160ROCK inhibitors (Fig. 5).

We also examined whether pharmacological interference with RhoA signaling could restore the motility deficit observed in Lis1-deficient neurons (Fig. 6). Exposure of cultured Lis1+/− neurons to inhibitors of the RhoA effector kinase p160ROCK had no effect on the total F-actin content (Fig. 6*F*). However, the inhibitors restored F-actin at the leading edge to wt control levels, (Fig. 6*H*) and restored filopodia number and length to wt values for both control and drug-treated conditions (Fig. 6*E*, *G*). These results confirmed that the modulation of Rho GTPases can eliminate cytoskeletal abnormalities associated with Lis1 deficiency.

This biochemical effect of pharmacological inhibition of RhoA was functionally associated with restoration to normal of *Lis1* +/− granule cell migration and of neurite elongation (Fig. 7). As shown in Figure 7*E*, compared with untreated wt neurons, the migration of *Lis1*+/− neurons along neurite fascicles was again significantly decreased at all distances from a cell cluster. In addition, the mean length of neurite fascicles extended from *Lis1*+/− cell aggregates was significantly shortened by 15% compared with *Lis1*+/+ neurons (Fig. 7*F*; $p < 0.0001$). Importantly, HA1077/Y27632 dramatically enhanced motility of *Lis1*+/− neurons along neurites, increasing the numbers of neurons at the farthest distances from the cluster to near wt levels (Fig. 7*E*, compare 7*A*,*B* and 7*C*,*D*). Interestingly, the number of either wt or *Lis1*+/− neurons found at distances of 0–50 μm from the cluster was little affected by the ROCK inhibitors. However, the number of Lis1-deficient neurons counted at 101–200 μm was greatly enhanced by the drug, nearing the treated wt control levels (Fig. 7*E*, *G*). Overall, suppression of Rho activity restored the distribution of treated Lis1+/− cells to most closely approximate the treated wt cells (Fig. 7*G*). These ROCK inhibitors also restored the axon length of the Lis1-deficient clusters to wt levels (Fig. 7*F*, compare 7*A*,*B* and 7*C*,*D*).

Lis1 deficiency interferes with motility and small GTPase activities in fibroblasts as well

Lis1 is a ubiquitously expressed protein, although the effects of its loss are most prominent in brain development. We, therefore, asked whether haploinsufficiency of *Lis1* in non-neuronal cells would produce a similar reduction in the activity of Rho GTPases and whether that would be reflected in a motility defect. As shown in Figure 8, *Lis1*+/− fibroblasts displayed a reduction of activated Rac1 to 46% (45.73 + 6.45%) and Cdc42 to 22% (22.13 + 0.33%) of wt levels ($p < 0.005$). Activated RhoA levels were increased in Lis1-deficient fibroblasts to 300% of control (291.36 + 26.05%; Fig. 8*D*). Unlike the fourfold reduction in motility of *Lis1*+/− neurons, however, fibroblasts monitored by time-lapse video microscopy displayed only a 30% decrease in average speed ($p < 0.0005$), compared with wt cells (Fig. 8*C*). Thus, the biochemical disturbance of Rho-GTPase activity was similar in Lis1-deficient neurons and fibroblasts. Interestingly, however, the degree to

which cell movement was impaired was far more dramatic for the neurite-guided neurons.

Discussion

Examinations of Lis1 function have focused on interactions with the microtubule-based cytoskeleton, with cytoplasmic dynein and with modulators of dynein. The present data demonstrate a defect in F-actin organization within Lis1-deficient neurons. Associated with this alteration are reduced GTP-Rac1 and GTP-Cdc42 activities and increased active RhoA. These altered relationships are the same in whole cerebellum and in isolated granule cells *in vitro*. The migration defect, reduced F-actin content at the leading edge, and axon elongation defect in cultured *Lis1*+/− cells are all restored by pharmacological interference with RhoA action and the consequent increased Rac1 and Cdc42 activity. Thus, in addition to previously described roles in regulation of microtubule function, Lis1 protein also promotes actin polymerization through signaling that suppresses RhoA activity, whether by direct or indirect mechanisms.

Coordinate abnormalities in *Lis1*+/− cells: impaired motility, defects in actin cytoskeleton, and disregulation of Rho GTPases

Time-lapse video microscopy of cerebellar granule cell migration demonstrated that glia-guided migration of Lis1-deficient cells was impaired. It also confirmed that motility of *Lis1*+/− granule neurons along neurites is impaired (Bix and Clark, 1998; Hirotsune et al., 1998). In the present study, this was caused by both decreased maximum velocity of movement and increased time spent at rest, suggesting that Lis1-deficient neurons have problems with both initiating movement and in their cycling through the steps of motility.

Another efficient measure of motility was the distribution of granule cells on neurites extended from reaggregate clusters after 24 hr in culture (Hirotsune et al., 1998). The number of granule neurons at a distance of ≥100 μm from the aggregate was dramatically lower in *Lis1*+/− granule cells. This assay in reaggregate clusters was validated by time-lapse video microscopic observations, and this simpler assay facilitated the evaluation of pharmacological manipulation on granule neuron motility.

Previous studies revealed Lis1 interactions with microtubules and with the dynein motor protein complex (Morris et al., 1998b; Horesh et al., 1999; Sapir et al., 1999; Caspi et al., 2000; Feng et al., 2000b; Sasaki et al., 2000; Smith et al., 2000; Han et al., 2001). However, the regulation of actin polymerization is another key component of cell motility (for review, see Lauffenburger and Horwitz, 1996). The altered appearance of filopodia and elaboration of a leading edge of migration during video microscopy prompted our examination of the actin cytoskeleton in Lis1-deficient neurons, using phalloidin staining. Indeed, the distribution of F-actin within the leading edge of neurons lacking Lis1 was abnormally low, suggesting altered regulation of actin polymerization in particular cell compartments. Because the F-actin cytoskeleton is a primary downstream target of Rho GTPases (Aspenstrom, 1999), we examined the small GTPases in *Lis1*+/− cells for alterations.

The present study provides the first indication that Lis1 is required to maintain physiological activities of the Rho GTPases that are central regulators of actin polymerization. Pull-down assays used GST fusion proteins to specifically bind active GTP-bound Rho, Rac1, and Cdc42. Relative to wild type, activated Rac1 and Cdc42 levels were reduced by 57% and 82%, respectively, at the same time

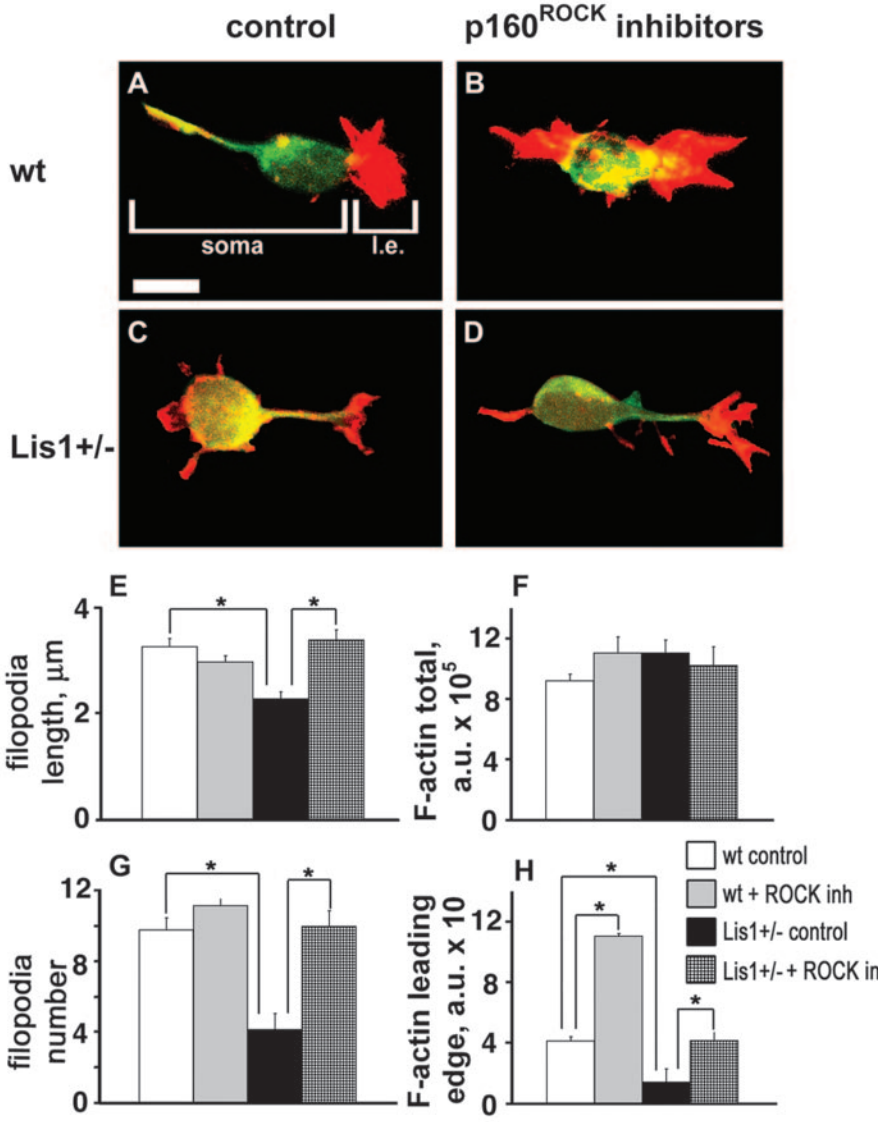

Figure 6. p160ROCK inhibitors rescue defects in the actin cytoskeleton of Lis1+/− neurons. *A–D*, Cultured cerebellar granule neurons stained with rhodamine–phalloidin (red) and βIII-tubulin (green). *A*, Wild-type neurons have a characteristic leading edge (l.e.) containing several filopodia and a cell body (soma) with little F-actin. *B*, Application of p160ROCK inhibitors to wt cells increases F-actin content in the leading edges. *C*, Control Lis1+/− neuron has a leading edge dramatically reduced in size, fewer filopodia, and an increased amount of F-actin in soma. *D*, Lis1+/− neurons treated with p160ROCK inhibitors look more like wt cells. Filopodia length (*E*) and number (*G*) are reduced in Lis1+/− cells ($n = 33$) compared with wt cells ($n = 41$), and p160ROCK inhibitors restore these parameters to wt values (wt, $n = 33$; Lis1+/−, $n = 33$). Whereas the total amount of F-actin labeling was unchanged (*F*), the amount of F-actin at the leading edge (*H*) was reduced in Lis1+/− cells. Scale bar, 10 μm. a.u., Arbitrary units. *$p < 0.01$.

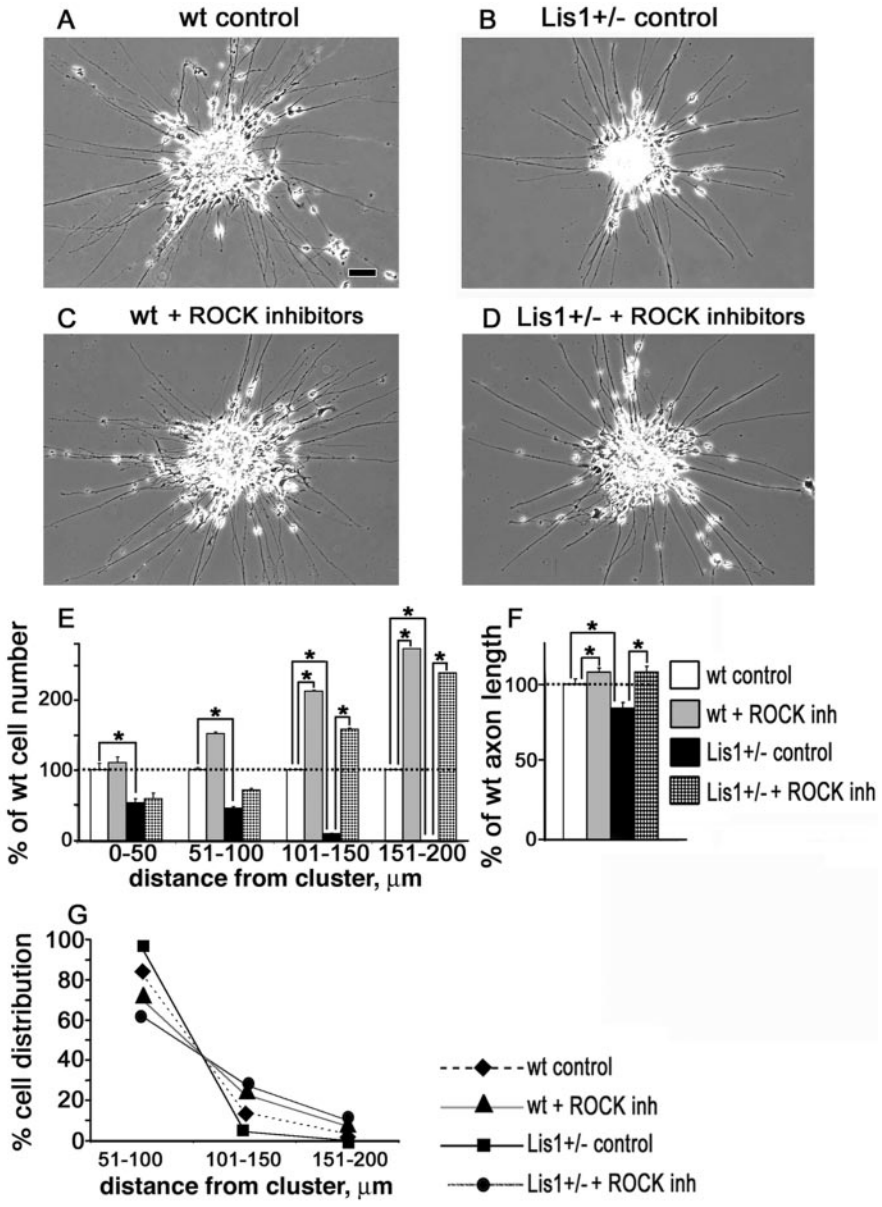

Figure 7. p160ROCK inhibitors enhance motility of wt cerebellar granule neurons and restore motility of *Lis1*+/− neurons. *A–D*, Reaggregate clusters of cerebellar granule neurons cultured on laminin for 24 hr. Treatment of granule neurons with p160ROCK inhibitors resulted in the enhanced migration out of the wt (*A* vs *C*) and Lis1+/− (*B* vs *D*) clusters. Treated Lis1+/− clusters (*D*) appeared more like wt clusters (*A*) with respect to the number of migrating neurons and length of axonal fascicles. *E*, Number of cells moved out from the clusters expressed as a percentage of wt control was greatly enhanced by 160ROCK inhibitors and brought untreated Lis1+/− to wt values ($n = 60$ clusters were analyzed for each genotype and treatment). *F*, Length of axonal fascicles was increased on p160ROCK inhibitor treatment in both wt neurons ($n = 847$ and 1032 axonal fascicles in control and treated groups, respectively) and Lis1+/− neurons ($n = 826$ and 1056 axonal fascicles in control and treated groups, respectively). *G*, Distribution of cells across 50 μm bins radiating from the Lis1+/− clusters was largely restored with more cells populating distant bins. Scale bar, 50 μm. *$p < 0.01$.

that GTP-bound RhoA was increased threefold in *Lis1*+/− cerebellar tissue, as well as in cultured neurons and fibroblasts. Thus, the disregulation of GTPases observed in Lis1-deficient cells was not an artifact of culture or migration substratum.

In *Lis1*+/− granule neurons, fluorescence labeling of F-actin in the leading margin was reduced whereas it was increased in the somitic compartment, so that total F-actin content was unchanged. This was consistent with the observed enhanced RhoA activity, because RhoA has been shown to antagonize Rac1/Cdc42-mediated actin polymerization (Grosheva et al., 2001). It is Rac1/Cdc42 that regulate actin polymerization at the leading edge of migrating cells (Nobes and Hall, 1995). Moreover, RhoA has been thought to promote the formation of contractile actin stress fibers, which may account for the enhanced F-actin labeling in granule cell somata.

Rac1 and Cdc42 promote the formation of the lamellipodia and filopodia, respectively (Nobes and Hall, 1995). The present analysis focused on filopodia, because they are easily recognized and reliably quantified. Consistent with the reductions in Cdc42 activity, *Lis1*+/− neurons contained 58% fewer filopodia, and average filopodial length was 30% shorter. Filopodia sample the environment of migrating neurons, and a 30% reduction in length translates into a 50% reduction in the potential sampling area for detection of guidance cues. This altered distribution of F-actin, the reduced filopodia number and length, support the significance of the disregulation of Rho GTPases caused by haploinsufficiency of *Lis1*.

Cross-talk between Rho GTPases

The particular regulatory relationships between Rho GTPases are cell type specific. In Swiss 3T3 fibroblasts, Rho GTPases have been placed in a hierarchical cascade in which Cdc42 activates Rac1, which activates Rho (Nobes and Hall, 1995). Activation of Rac1 by Cdc42 has also been shown in N1E-neuroblastoma cells and primary astrocytes (Kozma et al., 1997; Etienne-Manneville and Hall, 2001), and RhoA is activated by Rac1 in primary neurons (Li et al., 2002). In N1E-115 neuroblastoma, MCDK, NIH 3T3, and SV80 cells, Rac1 and Cdc42 inhibit RhoA activity, whereas in some cells this inhibition is mutual (Kozma et al., 1997; Leeuwen et al., 1997; Sander and Collard, 1999; Sander et al., 1999; Grosheva et al., 2001). RhoA also suppresses Rac1 activity in NGF-stimulated PC12 cells (Yamaguchi et al., 2001). Finally, in *Xenopus* optic tectal neurons, Cdc42 inhibits RhoA, and RhoA inhibits Rac1 (Li et al., 2002).

Here, the interdependence of Rho GTPases in cerebellar granule neurons was tested through inhibition of RhoA signaling. The direct inhibition of RhoA by C3 induced Rac1 and Cdc42 activities in wt neurons and approached wt activations in Lis1-deficient cells. Pharmacological inhibitors of p160ROCK induced twofold increases in GTP-bound Rac1 and Cdc42 in wild type and restored activity of Rac1 and Cdc42 to wt levels in *Lis1*+/− neurons (increases of 3.8-fold and 6-fold, respectively). Strikingly, restoration of Rac1 and Cdc42 activities by inhibition of RhoA signaling largely eliminated the defects in the actin cytoskeleton and neurite-guided neuronal migration. The relationship between overactivated RhoA and the downregulation of Rac1/Cdc42 was present in both *Lis1*+/− neurons and

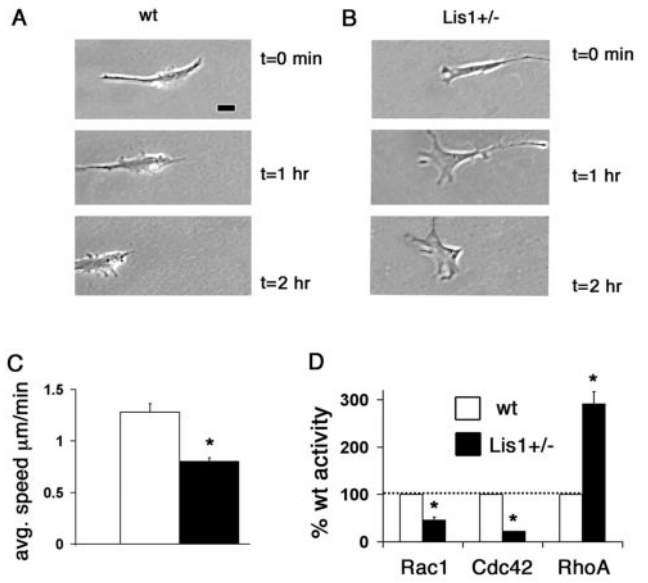

Figure 8. Like neurons, *Lis1*+/− fibroblasts exhibit motility defects and alterations in Rho GTPase activities. *A*, *B*, Wild-type fibroblasts ($n = 68$ cells; left) migrate faster on laminin than Lis1+/− fibroblasts ($n = 37$ cells; right), as shown by video-enhanced phase–contrast microscopy. *C*, Average speed of migration is reduced in Lis1+/− fibroblasts. *D*, Pull-down assay reveals decreased activity of Rac1 and Cdc42, whereas RhoA activity is increased ($n = 3$ experiments; three cultures of each genotype were analyzed per experiment). Scale bar, 10 μm. *$p < 0.01$.

fibroblasts. Furthermore, the effects of inhibition of RhoA or p160ROCK established the antagonism of Rac1/Cdc42 by RhoA activity in granule neurons.

Lis1 and the modulation of small GTPases in axon elongation

Rho GTPases play an essential role in axon growth, growth cone navigation, and the organization of F-actin in growing neurites (Kuhn et al., 2000). Axon growth in *Lis1*+/− neurons was, therefore, examined by measuring the length of axonal fascicles of cerebellar granule neurons after 24 hr in culture. Although the foreshortening of fascicles was not dramatic at 15%, it was nevertheless significant ($p < 0.0001$) and completely reversible with the agents used here. This again implicated Lis1 in modulating the actin-based cytoskeleton, because manipulation of RhoA and Rac1/Cdc42 fully restored normal measures.

Previous work has indicated a crucial role for Rho kinase (p160ROCK) in axon outgrowth of cultured granule neurons (Bito et al., 2000). In those studies, inhibition of p160ROCK accelerated axonal initiation and increased the size and motility of growth cones without changing the rate of axon elongation. The present data are consistent and suggest that increased RhoA activity in Lis1-deficient neurons is responsible for delays in axonal initiation. However, whether other factors, such as increased frequency of retraction, affects neurite growth in *Lis1*+/− neurons remain to be determined (Billuart et al., 2001).

Possible role of Lis1 in the coordinated regulation of cytoskeletal components.

Based on the data presented here and other published studies, two models for Lis1 function may be proposed (Fig. 9). In the first scenario, Lis1 interacts directly with RhoA to downregulate its activity and signaling to Rac1 and Cdc42 (Bishop and Hall, 2000). This would be consistent with the observation that cells with decreased Lis1 display higher levels of activated RhoA. In addition to its impact on actin polymerization, RhoA could influence

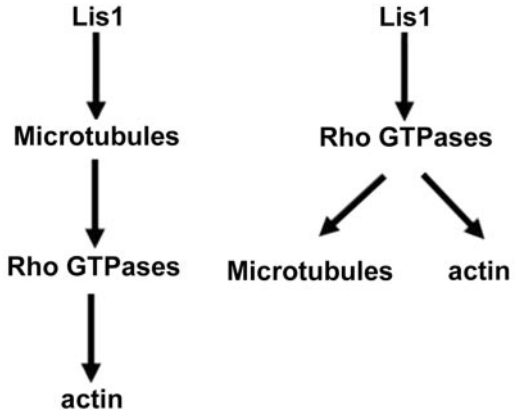

Figure 9. Possibilities for the interaction between Lis1 and Rho GTPases in migrating neurons.

microtubule dynamics. This mechanism would agree with previous reports that RhoA regulates microtubules by influencing the initial polarization of microtubules (Wittmann and Waterman-Storer, 2001). Thus, Lis1 could simultaneously influence microtubule organization and actin. We did not find evidence of direct Lis1–RhoA binding in Western pull-down assays. However, this does not rule out the possibility of low-affinity binding interactions that are not detectable in this method. More likely, Lis1 could interact with one or more of the numerous modulators of Rho GTPases (Kjoller and Hall, 1999), including the guanine nucleotide exchange factors, the GDP dissociation inhibitors, and GTPase activating proteins. Identification of such interactions will require extensive biochemical investigation beyond the scope of the present study.

An indirect influence of Lis1 on RhoA activity via Lis1 modulation of microtubules could also account for the present observations (Fig. 9). In this scenario, Lis1, which binds microtubules *in vitro* (Sapir et al., 1999), could act to modulate microtubule dynamics. In turn, microtubules have been shown to regulate RhoA activity, thereby influencing the actin cytoskeleton (Liu et al., 1998). In addition, microtubule growth has been suggested as a stimulus of Rac1 activity, thereby promoting protrusion of the leading edge of migrating cells via actin polymerization (Waterman-Storer et al., 1999).

Data presented here further enrich the emerging picture of the role of Lis1 in neuronal migration. The present studies indicate influences of Lis1 on the actin-based cytoskeleton, not just microtubule function, and suggest a role for Lis1 in the coordinate regulation of the actin and microtubule cytoskeletal components to promote cell motility. It remains to be determined whether these Lis1 influences on actin occur by direct signaling to Rho GTPases or are via microtubule-based signaling mechanisms.

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