A role for the lissencephaly gene *LIS1* in mitosis and cytoplasmic dynein function

Nicole E. Faulkner*, Denis L. Dujardin*, Chin-Yin Tai*, Kevin T. Vaughan†, Christopher B. O'Connell‡, Yu-li Wang‡ and Richard B. Vallee*§

*Departments of *Cell Biology and ‡Physiology, University of Massachusetts Medical School, 377 Plantation Street, Worcester, Massachusetts 01605, USA*
†Current address: Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556, USA
§e-mail: Richard.Vallee@umassmed.edu

Mutations in the *LIS1* gene cause gross histological disorganization of the developing human brain, resulting in a brain surface that is almost smooth. Here we show that LIS1 protein co-immunoprecipitates with cytoplasmic dynein and dynactin, and localizes to the cell cortex and to mitotic kinetochores, which are known sites for binding of cytoplasmic dynein. Overexpression of LIS1 in cultured mammalian cells interferes with mitotic progression and leads to spindle misorientation. Injection of anti-LIS1 antibody interferes with attachment of chromosomes to the metaphase plate, and leads to chromosome loss. We conclude that LIS1 participates in a subset of dynein functions, and may regulate the division of neuronal progenitor cells in the developing brain.

The migration of neurons from the progenitor zones of the developing central nervous system (CNS) to form the complex layers within the cortical regions represents an important, but incompletely understood, feature of brain development. A limited number of genes that control this process have been identified. Mutations in the human *LIS1* gene are responsible for type I lissencephaly (Miller–Dieker lissencephaly and isolated lissencephaly sequence), a condition characterized by gross disorganization of neurons within the cortical regions of the brain[1,2]. Genetic studies of patients with type I lissencephaly have revealed mutations in a single *LIS1* allele[3,4], and consequent reduction of levels of LIS1 protein[5,6]. Analysis of *Lis1*-knockout mice further supports the idea that reduced gene dosage gives rise to the lissencephalic phenotype[7]. Homozygous-null mice exhibit early embryonic lethality, indicating that LIS1 is also important in basic aspects of post-implantation cell physiology.

Two apparently distinct roles for LIS1 in neuronal migration have been proposed. First, LIS1 has been found to co-purify stoichiometrically with a brain cytosolic form of platelet-activating factor (PAF) acetylhydrolase 1b (PAFAH1b, ref. 8), an enzyme that inactivates PAF. A second potential role for LIS1 has been proposed on the basis of genetic studies. Mutations in an *Aspergillus LIS1* homologue, *nudF*, inhibit nuclear migration within hyphal processes[9], the same phenotype that is produced by mutations in the retrograde microtubule motor protein cytoplasmic dynein and its interacting partner, the dynactin complex[10–12]. Mutations in the yeast *LIS1* homologue *PAC1* produce defects in nuclear orientation[13], and mutations in *Drosophila Lis-1* produce dynein-like defects in nuclear migration and oogenesis[14,15].

In view of the disparate roles proposed for LIS1 and its importance in brain development, we sought to identify a role in the regulation of cytoplasmic dynein in higher eukaryotic cells. Here we present evidence for a physical interaction between LIS1, cytoplasmic dynein, and the dynactin complex, and co-localization at common subcellular sites. Perturbation of LIS1 levels in cultured mammalian cells has profound effects on mitotic progression, mitotic-spindle orientation and chromosome attachment, and on the subcellular localization of both cytoplasmic dynein and dynactin. Comparison with a dynactin phenotype that we previously characterized[16] reveals that LIS1 participates in a subset of the functions of cytoplasmic dynein. Together, these results indicate that LIS1 may have a fundamental function in the regulation of cytoplasmic dynein in cell division during the early stages of brain development.

Results

Co-immunoprecipitation of cytoplasmic dynein, dynactin and LIS1. To test for a physical interaction between LIS1 and cytoplasmic dynein, we immunoprecipitated LIS1 from cytosolic extracts of calf-brain tissue using two different antibodies specific for LIS1 (Fig. 1a). Both dynein and dynactin co-immunoprecipitated with LIS1 (Fig. 1b). LIS1 was also detected in dynein and dynactin immunoprecipitates (Fig. 1c). These results indicate that LIS1 exists as part of a complex with cytoplasmic dynein and dynactin.

Altered LIS1 expression affects cell division. As a further means of testing whether LIS1 participates in the function of dynein, we sought to perturb the expression of LIS1 in cultured mammalian cells. Our previous work has indicated that overexpression of the full-length wild-type dynamitin (p50) subunit of dynactin affects mitotic-spindle organization and causes cells to accumulate in a prometaphase-like state[16]. In addition, during interphase, the Golgi apparatus, endosomes, and lysosomes, are dispersed from their normal perinuclear locations[17].

Overexpression of LIS1 gave rise to an even more marked mitotic phenotype. Of MDCK cells transfected with a complementary DNA encoding full-length LIS1, one-third (mean mitotic index = 32.4%) were in mitosis, compared with 4.0 % for wild- type control cells (Fig. 2a). Cos-7 cells overexpressing LIS1 exhibited a mitotic index of 29.4%. The fraction of cells in prometaphase was increased relative to control cells (Fig. 2b), but not to the extreme extent observed in cells overexpressing dynamitin[16]. We also exposed Cos-7 cells to phosphothiolated LIS1-antisense oligonucleotides, which resulted in a 93% reduction in levels of LIS1 (Fig. 2c). Antisense-treated cells also accumulated in mitosis, although this effect was much less pronounced (a twofold increase in mitotic index) than for LIS1-overexpressing cells.

A range of defective mitotic structures could be observed in LIS1-overexpressing cells (Fig. 3a–f). The number of multipolar mitotic spindles increased in Cos-7 (8.1%, compared with 3.2% of

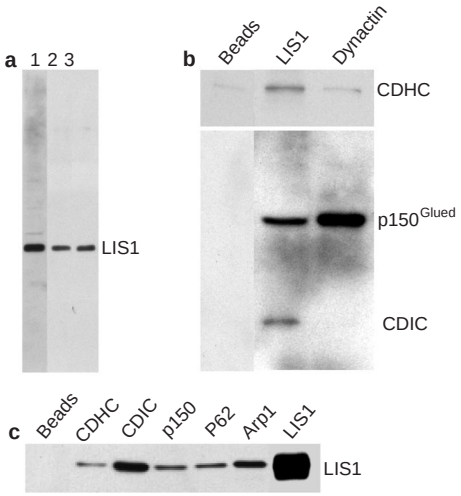

Figure 1 **Co-immunoprecipitation of cytoplasmic dynein and dynactin with LIS1. a**, Immunoblot of Cos-7-cell extracts. Lane 1, whole serum; lane 2, immunoglobulin G fraction; lane 3, affinity-purified anti-LIS1 antibody produced during this study. **b**, LIS1 and p150^{Glued} (Dynactin) were immunoprecipitated from bovine-brain cytosol and immunoblotted using antibodies against the heavy (CDHC) and intermediate (CDIC) chains of cytoplasmic dynein and against p150^{Glued}. All three polypeptides were enriched in the LIS1 immunoprecipitate, although a limited amount of dynein heavy chain was present in controls (Beads). **c**, The heavy- and intermediate-chain subunits of cytoplasmic dynein, the p150^{Glued}, p62 and Arp1 subunits of dynactin, and LIS1 were immunoprecipitated and immunoblotted using anti-LIS1 antibody. LIS1 co-immunoprecipitated with all dynein and dynactin antibodies tested.

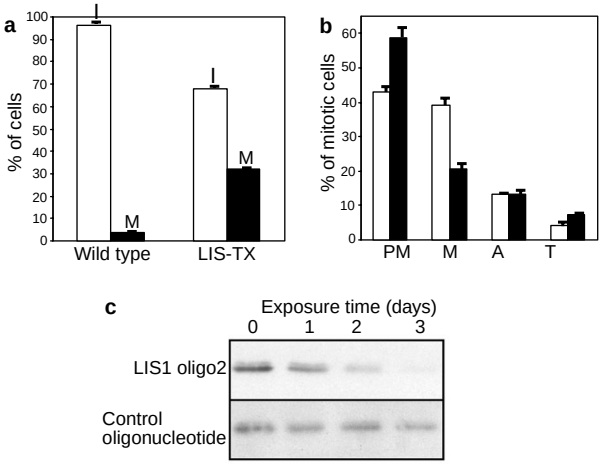

Figure 2 **Overexpression of LIS1 perturbs mitotic progression. a**, Percentages of cells in mitosis (M) and interphase (I) in control wild-type or LIS1-overexpressing (LIS-TX) MDCK cultures. Values are means ± s.d. from three experiments. **b**, Percent of LIS1-overexpressing MDCK cells in prometaphase (PM), metaphase (M), anaphase (A) and telophase (T). Open bars, wild-type controls; filled bars, LIS-1-overexpressing cells. Values are means ± s.d. from four experiments. **c**, Western blot, using anti-LIS1 polyclonal antibody, of extracts from Cos-7 cells exposed to 100 nM oligo2 (see Methods) and to control phosphothiolated oligonucleotides.

total mitotic cells) and MDCK (9.5%, compared with 2.7%) cells. Supernumerary spindle poles contained centrioles, as detected with an anti-polyglutamylated tubulin antibody[18,19], which is consistent with a delay in mitosis (data not shown, see Discussion). An increase in multipolar spindles was also observed in antisense-treated cells (9.5%, compared with 1.5% of total mitotic cells). LIS1-overexpressing cells with otherwise well-formed metaphase plates exhibited a greater-than-normal number of unaligned chromosomes (Fig. 3c, f), suggesting a defect in chromosome congression or attachment. A very high percentage of interphase cells contained micronuclei and/or multiple nuclei (43.3%, compared with 8.9% for control cells), which is consistent with a lack of proper chromosome segregation.

Many mitotic LIS1-overexpressing cells were unusually flat and well-spread (see, for example, Fig. 3c), and had randomly positioned spindles, often with one pole flush against the cortex (Fig. 3c). In some late-prometaphase and metaphase cells (Fig. 3m, n), hyperelongated astral microtubules were seen to reach the cell cortex, where they bent either along the cell margin or back into the cytoplasm. This pattern is similar to that observed in dynein-, dynactin- and *PAC1*-knockout yeast strains[13,20], indicating that overexpression of LIS1 in mammalian cells may interfere with a similar interaction.

In contrast to the pronounced effects observed during mitosis, overexpression of LIS1 had no effect on the distribution of the Golgi apparatus, endosomes or lysosome during interphase (Fig. 3g–l).

LIS1 overexpression alters the distribution of cytoplasmic dynein and dynactin. Dynein and dynactin have been implicated in the attachment of microtubules to the cell cortex in *Saccharomyces cerevisiae*[20] and *Caenorhabditis elegans*[21,22]. To investigate the function of LIS1 in this interaction, we examined the effects of LIS1 overexpression on the distribution of cytoplasmic dynein and dynactin in MDCK cells. In control cells, dynein and dynactin staining was observed at the cell cortex, as has been reported[23], along spindle and astral microtubules, and at spindle poles (Fig. 4a). In LIS1-overexpressing cells, dynein (data not shown) and dynactin were still readily detected at the latter sites (Fig. 4b). However, cortical staining was clearly disrupted and replaced with disorganized clumps that were randomly situated at the cell cortex and in the cytoplasm. In turn, the astral microtubules no longer radiated in a uniform pattern from the centrosomes, but rather were focused towards the cortical clumps (Fig. 4b).

These observations support the idea that LIS1 functions in the regulation of the distribution of dynein and dynactin in the cortex, and their interaction with astral microtubules. To determine whether LIS1 affects spindle orientation, we examined filter-grown, polarized MDCK cells. Mitotic spindles in these cultures are almost all situated parallel to the substratum[24] (Fig. 4c). In LIS1-transfected cells, the orientation of the spindle axis was randomized (Fig. 4d); 62.5% of bipolar mitotic spindles were found to have their two poles in different focal planes, compared with 15.0% for controls. Polarized MDCK cells have been reported to exhibit a restricted pattern of cortical dynein and dynactin staining in a belt just below the tight junctions[23]. In polarized MDCK cells overexpressing LIS1, dynein (data not shown) and dynactin were again observed to form small clumps that were dispersed throughout the cell (Fig. 4f).

Function of LIS1 and cytoplasmic dynein at the kinetochore. The observation of unaligned chromosomes in LIS1-overexpressing cells and the accumulation of cells in mitosis (Figs 2 and 3) are consistent with a defect in chromosome congression or in attachment of chromosomes to mitotic-spindle microtubules. Surprisingly, however, the immunoreactivity of cytoplasmic dynein and dynactin persisted at prometaphase kinetochores of LIS1-overexpressing cells (Fig. 4g). To determine more directly whether LIS1 indeed participates in kinetochore function, we microinjected anti-LIS1 antibody into NRK cells just before breakdown of the nuclear envelope. We observed the effects on chromosome behaviour from early prometaphase onwards, and observed clear delays in progression through mitosis (Fig. 5 and Supplementary Information). Immediately after nuclear-envelope

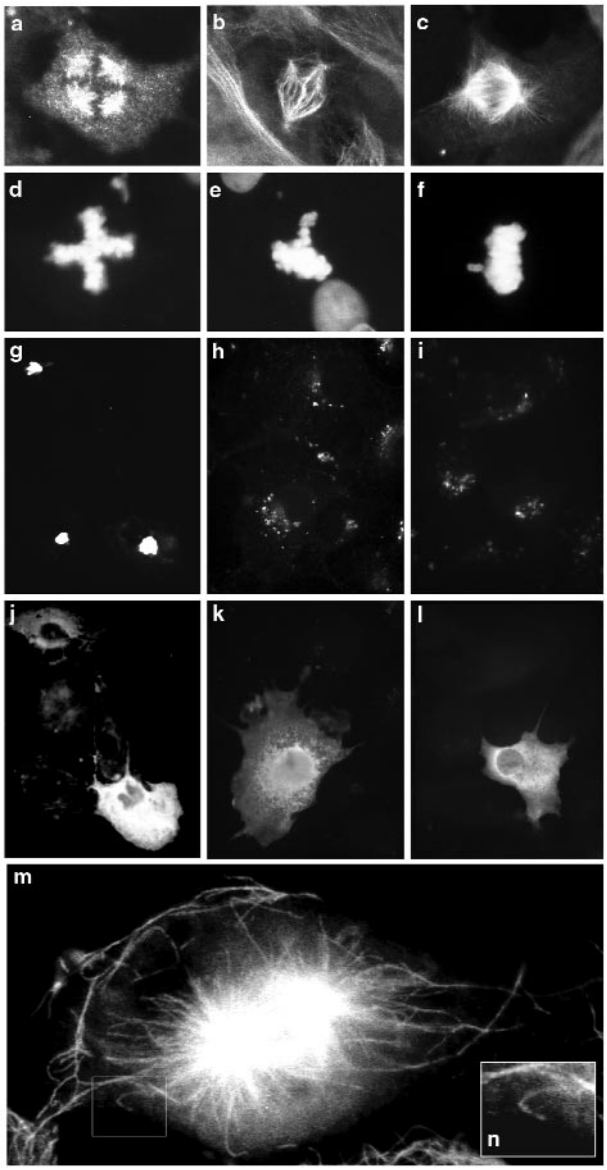

Figure 3 **Phenotypic effects of LIS1 overexpression. a–f**, Immunofluorescence-microscopic images of LIS1-overexpressing Cos-7 cells stained with anti-tubulin antibody (**a–c**) and Hoechst (**d–f**) to reveal chromosomes. **a**, **b**, **d**, **e**, Tetrapolar and split polar spindles; **c**, **f**, bipolar spindle with misaligned chromosomes. Note that the left spindle pole lies against the cell cortex. **g–l**, Double-labelling using GFP–NAGT (**g**), RITC–transferrin (**h**) or lysotracker (**i**); anti-myc antibody was used to identify LIS-1-overexpressing cells (**j–l**). No effect was observed on the distribution of vesicular organelles. **m**, Prometaphase cell stained with anti-tubulin antibody, showing hyperelongated microtubules that curve back from the cell cortex. **n**, box marked in **m**, shown at higher magnification.

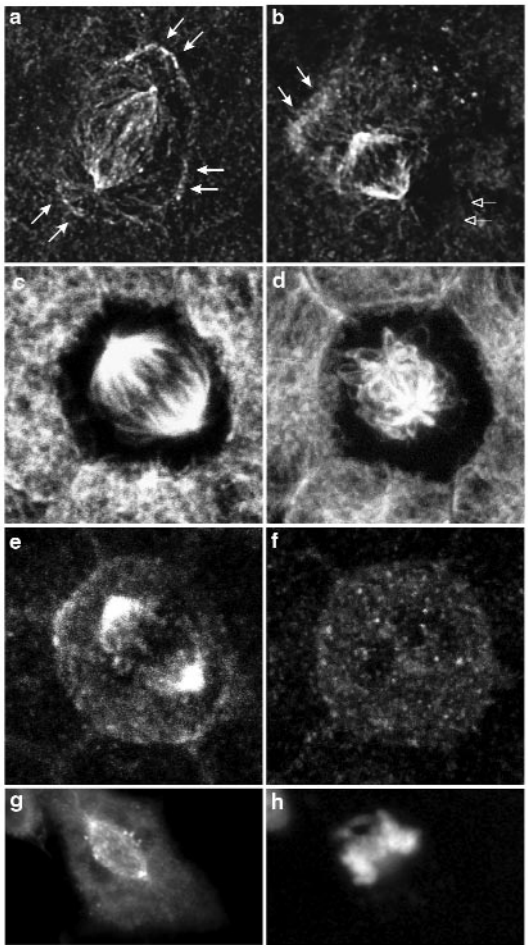

Figure 4 **Effects of LIS1 overexpression on dynactin distribution. a**, **b**, Confocal-microscopic images of mitotic control (**a**) and LIS1-overexpressing (**b**) MDCK cells stained with anti-p150^{Glued} antibody. Punctate cortical staining is present in the control cell (filled arrows). In the LIS1-overexpressing cell, cortical staining is largely disrupted (open arrows), although some remains (filled arrows). **c–f**, Confocal-microscopic images of filter-grown, polarized MDCK cells stained with anti-tubulin (**c**, **d**) anti-p150^{Glued} (**e**, **f**) antibodies. The spindle in the control, non-transfected cell (**c**, **e**) is parallel to plane of the coverslip, whereas that in the LIS1-overexpressing cell (**d**, **f**) is severely tilted. **e**, **f**, Centre panels from a confocal z-axis series, showing dynactin at the cortex and poles of the control cell (**e**), but dispersed in the LIS1-overexpressing cell (**f**). **g**, **h**, Labelling of LIS1-overexpressing, non-polarized MDCK cells with anti-p150^{Glued} (**g**) and a DNA-labelling compound (**h**), showing persistent dynactin staining at prometaphase kinetochores.

breakdown, condensed chromosomes showed a tendency to disperse throughout the cell and then to follow a less orderly pattern of migration to the metaphase plate than was observed in controls. In ~50% of experimental cells (8/17), chromosomes exhibited delayed alignment with the metaphase plate, or, once aligned, diffused away, indicating that alignment was unstable. In each case, repeated approaches to the plate were observed, suggesting multiple attempts at chromosome recapture or reorientation. This behavior resulted in a delay in anaphase onset. In some cells, chromosomes rejoined the metaphase plate before anaphase onset (9/17), whereas in the remainder (8/17), anaphase proceeded with an unattached or mono-oriented chromosome (Fig. 5f, m) leading in some cases to the formation of micronuclei (Fig. 5j, o). Several cells showed stretched chromosomes during anaphase (5/17). Although prometaphase was clearly extended in cells injected with anti-LIS1 antibody, the duration of metaphase, anaphase, and telophase were unaffected.

These results are consistent with a functional relationship between LIS1 and cytoplasmic dynein and dynactin. These proteins have been predicted to participate in chromosome capture and attachment on the basis of their prominent localization at the kinetochore during prometaphase[16,25,26] and the observation of rapid poleward chromosome movements during early prometaphase in some cells[27]. However, direct evidence that dynein and dynactin are involved in chromosome movement and

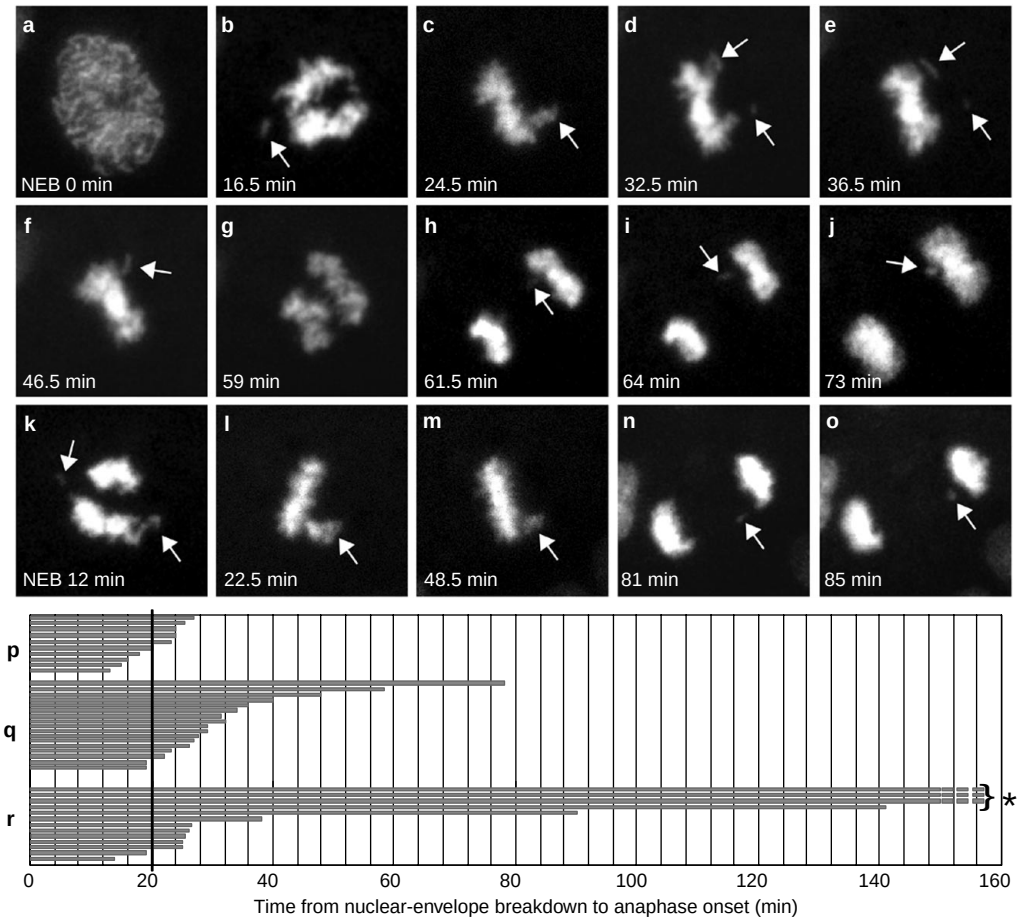

Figure 5 **Effects of anti-LIS1 antibody on the behaviour of mitotic chromosomes. a–o**, Time-lapse images, obtained at the indicate times after nuclear-envelope breakdown (NEB), of two NRK cells stained with Hoechst 33258 to visualize chromosomes, and injected with anti-LIS1 antibody just before nuclear-envelope breakdown. **a–j**, In one example, chromosomes can be seen joining and dissociating from the metaphase plate (arrows), one of which is lost during anaphase and forms a micronucleus. **k–o**, Another example, showing formation of micronuclei. **p–r**, Summary of the effects of antibody microinjection. Horizontal bars represent time from nuclear-envelope breakdown until anaphase onset. Vertical bar indicates the mean value for controls (20.1 min). **p**, Control injections (pre-immune immunoglobulin G fraction of or LIS1-peptide-blocked antibody). **q**, Cells injected with anti-LIS1antibody showed clear delays, although all cells eventually initiated anaphase. **r**, Cells injected with a monoclonal antibody against the intermediate chain of cytoplasmic dynein exhibited a more pronounced prometaphase delay. Several cells remained in prometaphase for >2 h (*), after which time they were no longer monitored.

attachment has been elusive. To test for such a role and for comparison with LIS1, we injected NRK cells with an antibody against the cytoplasmic-dynein intermediate chain. The anti-dynein antibody caused an even more pronounced prometaphase delay than that observed with anti-LIS1 antibody, with several cells remaining in prometaphase for ≥2 h (Fig. 5p–r). Chromosomes in several anti-dynein-injected cells (7/13) were repeatedly lost and realigned at the metaphase plate. No examples of anaphase with unattached chromosomes were observed.

Subcellular distribution of LIS1. We observed no evidence for an association of LIS1 with interphase microtubules by immunofluorescence microscopy or by microtubule co-purification[28], in contrast to previous findings[29], although we did see staining of mitotic microtubules when we used one polyclonal antibody. Both this and another monoclonal antibody[29] recognized prominent chromosome-associated spots (Fig. 6a–c). These spots were identified as kinetochores by co-localization with anti-dynamitin antibody (Fig. 6g–i). As for dynein and dynactin, LIS1 immunoreactivity at kinetochores was most prominent during prometaphase and was greatly reduced or absent in attached chromosomes during late prometaphase and metaphase, which is consistent with the distribution of cytoplasmic dynein[25,26] and dynactin[16]. Clear staining of the cell cortex was also observed in MDCK cells with both anti-LIS1 antibodies (Fig. 6a–f).

Role of LIS1 in regulating proteins associated with microtubule plus ends. Several proteins have been identified that associate with the distal (plus) ends of growing cytoplasmic microtubules[30–33], including dynactin (ref. 34 and K.T.V., unpublished observations). How this behaviour relates to the interaction of microtubules with the cell cortex is unknown, but we sought to determine whether it was affected by overexpression of LIS1. In control cells, the p150^{Glued} subunit of dynactin co-localized with CLIP-170 (ref. 34) and EB-1 (Fig. 7a–f), a dynactin- and adenomatous polyposis coli (APC)-interacting protein[35]. Overexpression of LIS1 virtually abolished p150^{Glued} staining from microtubule ends (Fig. 7g, h). However, no effect on EB-1(Fig. 7i, j) or CLIP-170 (data not shown) was observed.

Discussion

Our immunoprecipitation experiments have demonstrated a physical interaction between cytoplasmic dynein and LIS1, although we

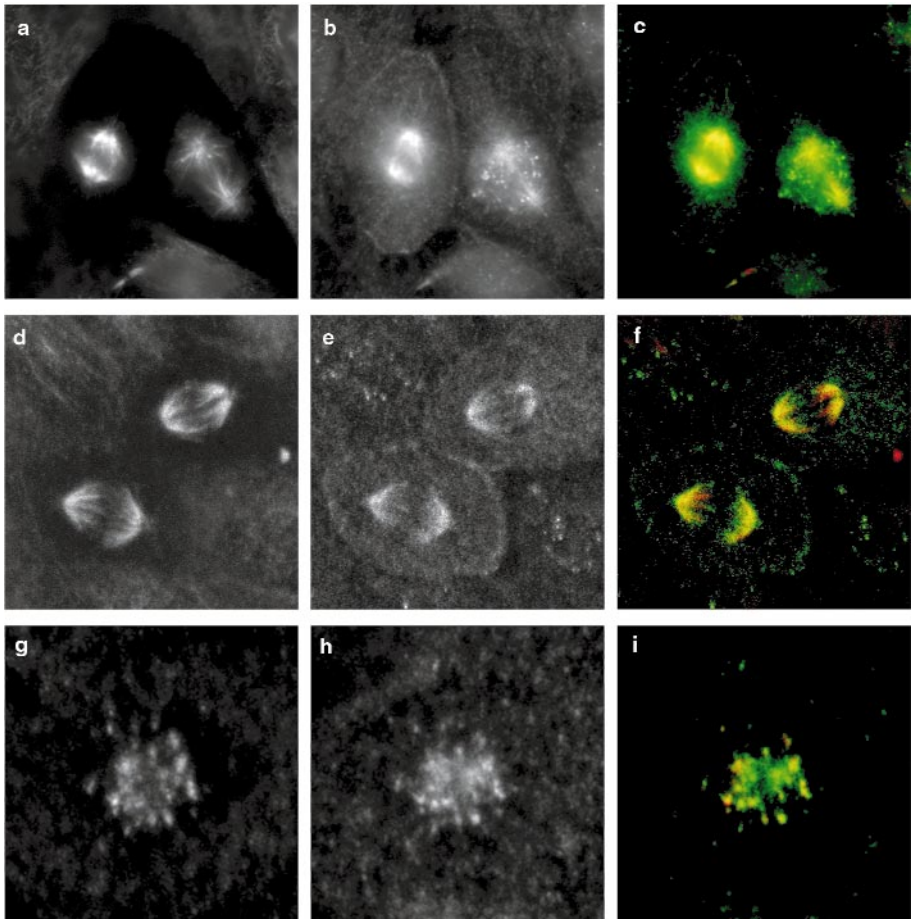

Figure 6 **Subcellular localization of LIS1.** Immunofluorescence-microscopic images of MDCK cells stained with anti-tubulin antibody (**a**, **d**; red in overlay), affinity-purified anti-LIS1 polyclonal antibody (**b**, **e**, **h**; green in overlay) or anti-dynamitin antibody (**g**; red in overlay). **a–c**, In early prometaphase, LIS1 is localized to both kinetochores and spindle poles. **d–f**, In metaphase, LIS1 is present at the cortex (confocal images). **g–i**, LIS1 co-localizes with dynamitin at prometaphase kinetochores.

do not know whether binding is direct or indirect. The co-precipitation of both dynein and dynactin is of some interest, in that the two complexes do not generally co-immunoprecipitate with each other[36], with the exception of the triple complex of dynein, dynactin and NuMA[37]. Our results indicate the possible existence of another such complex involving LIS1. It is appealing to speculate that these supercomplexes reflect an activated state of dynein, which may be involved in subcellular targeting, on the basis of existing evidence regarding the role of dynactin in dynein function[16,38].

The results of our localization studies are consistent with an interaction between LIS1 and the dynein–dynactin complex. We found LIS1 to be associated with the cortex of mitotic MDCK cells (Fig. 6), where cytoplasmic dynein and dynactin are also present[23] (Fig. 4). These results are consistent with a recent report that *Drosophila* Lis-1 and cytoplasmic dynein both associate with the cortices of *Drosophila* oocytes[14]. Remarkably, we also found LIS1 to be present at the kinetochore during prometaphase (Fig. 6). The intensity of LIS1 staining declined through subsequent stages of mitosis. This behaviour is identical to that observed for cytoplasmic dynein and dynactin, providing further support for a common site of action.

The effect of LIS1 overexpression on the localization of dynein and dynactin adds further support to the idea of a physiological interaction. We found that excess LIS1 alters the distribution of both complexes at the cortices of dividing MDCK cells (Fig. 4) and affects the association of dynactin with microtubule plus ends (Fig. 7). Curiously, there is no clear effect on the association of dynein or dynactin with kinetochores (Fig. 4), indicating that, at least in this case, LIS1 may have a function in regulating the activity of cytoplasmic dynein rather than cargo attachment.

The differential effects of LIS1 overexpression on the association of p150^{Glued}, EB-1 and CLIP-170 with microtubule distal ends provide further evidence for a specific function for LIS1 in the dynein–dynactin pathway. Furthermore, they indicate that the loss of p150^{Glued} from microtubule ends is not simply a result of stabilization of microtubules, which abolishes the end-binding of CLIP-170 (ref. 32), EB-1 (ref. 31) and p150^{Glued} (N.E.F. *et al.*, unpublished observations). Finally, these results further indicate that excess LIS1 interferes with the interaction between EB-1 and dynactin.

Type I lissencephaly affects only the brain, suggesting a function for the LIS1 protein in neuronal development. However, LIS1 is expressed in many tissues[2], and homozygous-null mice exhibit early embryonic lethality[7], which is consistent with a more general function in cell physiology, as we report here. Two of the approaches we used — antibody injection and exposure to antisense oligonucleotides — mimic the disease state by reducing active protein levels. The LIS1-overexpression phenotype may result from a competition mechanism, as seems to be the case for dynamitin[16,39]. Sequestration of dynein or dynactin by overexpressed LIS1 seems a less likely mechanism in view of the persistence of the two complexes at the prometaphase kinetochore (Fig. 4g, h).

The effects produced by the three different approaches we used

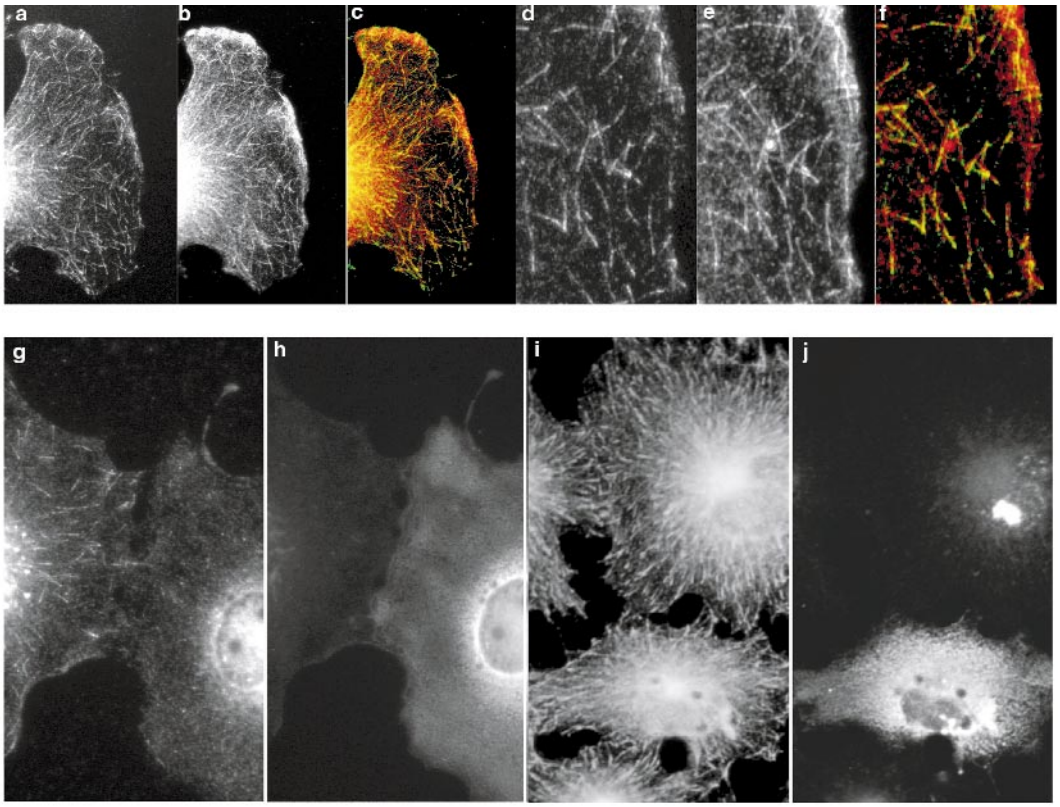

Figure 7 **Overexpression of LIS1 displaces p150^{Glued} from microtubule plus ends. a–f**, Immunofluorescence-microscopic images of control interphase Cos-7 cells treated with anti-p150^{Glued} (**a**, **d**; red in **c**, **f**) and anti-EB-1 (**b**, **e**; green in **c**, **f**) antibodies, showing staining of identical microtubule ends. **g–j**, Loss of dynactin, but not of EB-1, from microtubule distal ends in LIS1-overexpressing cells. LIS1-overexpressing Cos-7 cells were identified with anti-Myc antibody (**h**, **j**) and double-labelled with anti-p150^{Glued} (**g**) or anti-EB-1(**i**) antibodies.

are complementary, and are consistent with a function of LIS1 at the two primary sites where we found it to be localized, the cortex of the mitotic cell and the kinetochore (Fig. 8). Several lines of evidence point to a defect in the interaction between mitotic-spindle microtubules and the cell cortex. Most direct is the misorientation of mitotic spindles in polarized MDCK epithelial cells (Fig. 4). Cytoplasmic dynein has been reported to form a cortical belt around these cells at the level of the mitotic spindle[23], perhaps actively holding it in place. The randomization of spindle orientation we observed in LIS1-overexpressing cells is associated with dispersal of dynein and dynactin (Fig. 4), which strongly suggests that the spindles are no longer properly attached. These spindles do not appear to be entirely free in the cytoplasm, as we still observed microtubules extending to sites at the cell periphery. It is possible, therefore, that it is the disorganization of sites for linkage of dynein to microtubules within the cortex, rather than complete detachment of microtubules, that is responsible for spindle misorientation. A defect in the interaction between microtubules and the cell cortex is further indicated by the extended, curved astral microtubules we observed during prometaphase in LIS1-overexpressing Cos-7 cells (Fig. 3). This pattern is reminiscent of that observed for cytoplasmic microtubules in yeast cells with defective cytoplasmic dynein, dynactin and *PAC1* (refs 13, 20). It is also worth noting that cytoplasmic-dynein-mediated defects in mitotic-spindle orientation can contribute to mitotic delay in yeast[40]. More recently, spindle displacement in mammalian cultured cells has also been found to delay mitotic progression[41]. Thus, the defects we observed in spindle orientation may contribute to the increased mitotic index in LIS1-overexpressing cells (see below).

Our data also clearly point to a role for LIS1 in kinetochore function. We observed clear localization of LIS1 to these structures (Fig. 6), which was temporally coincident with that of cytoplasmic dynein and dynactin. A substantial number of LIS1-overexpressing cells with otherwise well-formed metaphase plates exhibited unaligned chromosomes (Fig. 3), and clear delays in alignment were observed in antibody-injected cells (Fig. 5). Chromosomes were also lost from the metaphase plate with some frequency, and cycles of capture and loss were observed, behaviour that was not seen in control cells. It is likely that the resulting delays in mitosis contribute significantly to the increased mitotic index we observed in LIS1-overexpressing cells. The increase in multipolar cells in the transfected cultures (Fig. 3) and in cells exposed to antisense oligonucleotides (data not shown) are also consistent with a defect in mitotic progression . The immunoreactivity of the supernumerary poles with anti-polyglutamylated tubulin antibody, a marker of centrioles, indicates that the extra poles may have resulted from premature splitting of the centriole pairs, or from an extra round of centrosome replication, effects that are observed after extensive mitotic delays[42].

Cytoplasmic dynein and dynactin have been implicated in the attachment of chromosomes to mitotic microtubules, on the basis of the localization of the two complexes to prometaphase kinetochores[16,25,26], the poleward movement of sister-chromatid pairs during early prometaphase[27], and the prometaphase block caused by overexpression of dynamitin, which also dissociates dynein and dynactin from the kinetochore[16]. A mutation in the *Drosophila* dynamitin-interacting kinetochore protein *zw10* also releases dynein and dynactin from kinetochores, but results in loss of chromosomes during anaphase[39]. Whether the latter effect involves dynein is uncertain, as *zw10* persists at the kinetochore during anaphase after dynein levels have decreased.

We observed a clear defect in chromosome alignment in cells

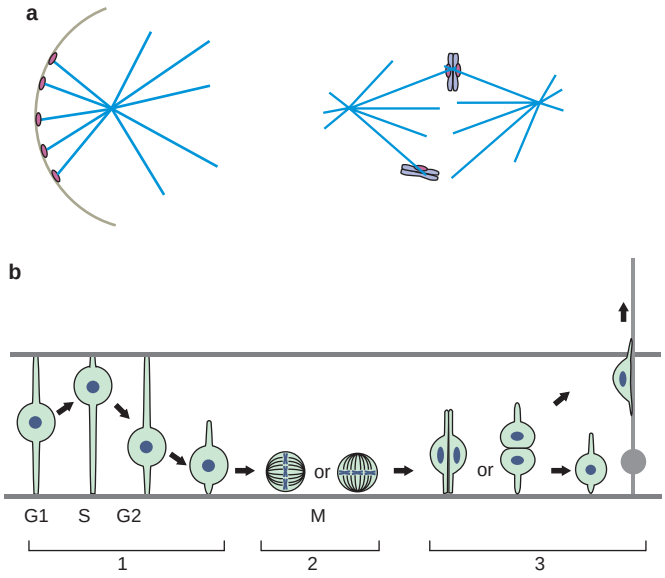

Figure 8 **Potential functions of LIS1 in mitosis and brain development. a**, Proposed functions of LIS1 in mitosis. LIS1 (pink) is depicted as participating in the cytoplasmic-dynein-mediated interaction between microtubules and kinetochores (right) and between microtubules and the cell cortex (left). **b**, Possible functions of LIS1 in brain development. Neuronal-progenitor cells in the vertebrate ventricular zone are shown exhibiting cell-cycle-dependent nuclear oscillations (1), during mitosis (2) and after cell division (3). For cells dividing parallel to the ventricular plane, one of the progeny remains undifferentiated, as shown, whereas the other differentiates and migrates outwards along radial glial fibres. In addition to potential functions of LIS1 during phases 1 and 3, our results points to important roles for LIS1 in mitotic progression and spindle orientation, both of which may profoundly affect the subsequent distribution of neurons within the brain. (**b** is after refs 45, 49).

injected with anti-dynein antibody (see Supplementary Information), very similar to the results of injection with anti-LIS1 antibody, which, however, produced a less pronounced mitotic delay. This difference may result from the tendancy of anti-LIS1-injected cells to proceed through anaphase with unattached chromosomes, behaviour that we have so far not observed in anti-dynein-injected cells. This behaviour is potentially of considerable importance, as it indicates that loss of LIS1 function may specifically affect the mitotic-checkpoint mechanism. That the chromosome-attachment checkpoint is still partially operational in these cells is indicated by the observed prometaphase delays. However, that the checkpoint is defective is also clear from the premature entry into anaphase and resulting loss of chromosomes, as observed in *zw10*-mutant flies[43]. Together, therefore, these data indicate that dynein and its associated proteins may function not only in chromosome attachment, but also in the checkpoint mechanism itself.

Overexpression of LIS1 had no apparent effect on the cytoplasmic-dynein-mediated localization of vesicular organelles (Fig. 3). Thus, the effects we observed on cortical and kinetochore interactions during mitosis could mean that LIS1 is only functional during mitosis. However, this possibility seems unlikely in view of evidence for non-mitotic functions of LIS1-related proteins in lower eukaryotes[9,14,15], and our evidence for an effect of LIS1 overexpression on the composition of microtubule ends (Fig. 7). Alternatively, we note that the LIS1 functions revealed here may have as a common theme the interaction between cytoplasmic dynein and dynactin and microtubule plus ends (Fig. 8a). Perhaps, therefore, LIS1 functions as a novel regulator of microtubule-end-dependent processes.

Our findings have implications for understanding the development of the lissencephalic brain. Cell division occurs primarily in the neuroepithelial layers of the early CNS, from which differentiating neurons emerge and migrate to their final destinations (Fig. 8b). LIS1 and cytoplasmic dynein could conceivably participate at any of several stages during this process. First, they could function in the oscillatory movements of nuclei during the cell-division cycle of neuronal progenitor cells in the ventricular zone (Fig. 8b, 1). This behaviour is reminiscent of the dynein- and *nudF*-dependent nuclear migration observed in growing *Aspergillus* hyphae, and possibly of the basolateral–apical shift in nuclear position in the developing *Drosophila* eye, a process that seems to be partially impeded in a *Lis1*-null mutant[14]. LIS1 could also participate directly in post-mitotic neuronal migration (Fig. 8b, 3). This possibility is supported by a reduction in the outward migration of neurons from *in vitro* aggregates, using cells from Lis1-mutant mice[7]. How this form of motility relates to the glial-cell-guided process *in vivo* and to the function of cytoplasmic dynein is uncertain.

Our data indicate a role for *LIS1* in cell division, alterations in which could, in turn, affect neuronal migration, as we have previously speculated[46]. Neuroepithelial cells undergoing cleavage perpendicular to the ventricular plane give rise to progeny that continue to divide (Fig. 8b, 2). Cells undergoing cleavage parallel to the ventricular plane produce non-equivalent progeny, one of which continues to divide while the other differentiates and undergoes migration[45]. For the differentiating neuron, the timing of the last division (its 'birthday') affects its final destination by a mechanism that remains mysterious[46]. In view of these observations, it seems reasonable to expect that mutations that influence the orientation of the cell-division plane or the timing of cell division may affect the ultimate distribution of neurons in the brain. Our data identify a role for LIS1 in regulating these processes, and indicate that defects in cell division may contribute to lissencephaly. □

Methods

cDNAs and immunological reagents.

A full-length LIS1 cDNA was generated by polymerase chain reaction with reverse transcription (RT-PCR) from Cos-7-cell RNA, using primers directed to conserved flanking UTR sequences. The amplified cDNA was sequenced and subcloned into the pCMV mammalian expression vector with an amino-terminal myc epitope (Clontech). Two polyclonal rabbit antibodies were made to a peptide corresponding to the first 21 amino acids of human LIS1 conjugated to keyhole-limpet haemocyanin (KLH) (Research Genetics, Huntsville, Alabama). One of the antibodies was affinity-purified against peptide conjugated to sepharose 4b beads (Research Genetics), or used as an immunoglobulin G fraction prepared by binding and extraction from protein G–sepharose (Amersham) using 100 mM glycine, pH 3.0. Other polyclonal and monoclonal antibodies against human LIS1 (ref. 29) were provided by O. Reiner (Weizmann Institute, Rehovot, Israel). Antibodies against dynein and dynactin included a polyclonal[47] and a monoclonal (Transduction Laboratories) anti-p150^{Glued}, monoclonal anti-dynamitin[36], monoclonal antibodies against cytoplasmic-dynein intermediate chain (74.1, gift from K. Pfister, Univ. Virginia, Charlottesville, Virginia; and 70.1, Sigma), polyclonal antibody against cytoplasmic-dynein intermediate chain (L5, K.T.V.), polyclonal antibody against cytoplasmic-dynein 1 heavy chain (gift from A. Mikami, Univ. Massachusetts Medical School, Worcester, Massachusetts), polyclonal anti-p62 (ref. 48), and polyclonal anti-Arp1 (gift from D. Meyer, UCLA, Los Angeles, California). Other antibodies included monoclonal antibody against polyglutamylated tubulin GT335 (ref. 23, gift from P. Denoulet, College de France, Paris), monoclonal anti-EB1 (Calbiochem), monoclonal anti-CLIP-170 (gift from H. Goodsen, Univ. Notre Dame, Notre Dame, Indiana), and monoclonal anti-tubulin (DM1A, Amersham).

Immunoprecipitation.

LIS1, cytoplasmic dynein and dynactin were immunoprecipitated from calf-brain cytosol[36] using affinity-purified polyclonal anti-LIS1 and monoclonal anti-LIS1 antibodies, polyclonal antibodies against cytoplasmic-dynein intermediate chain and heavy chain, and polyclonal anti-p150^{Glued}, anti-p62 and anti-Arp1 antibodies bound to protein G–Sepharose beads (Amersham). Immunoprecipitates were dissolved in sample buffer, resolved by SDS–PAGE, and transferred to polyvinylidene-difluoride membrane (Immobilon P, Millipore). Western blotting was carried out as described[16].

Cell culture, transfection and antisense.

Cos-7 and NRK cells (ATCC nos CRL1651 and NRK-52E, respectively) were maintained as described[16,41]. MDCK cells were from J. de Mey (Institut Curie, Orsay, France) and were maintained as described[23]. To generate polarized MDCK cells, cultures were grown to confluence on Anopore™ membrane in Nunc Tissue Culture inserts (Nalge Nunc International, Naperville, Illinois). For transient transfections, Cos-7 and MDCK cells were plated on coverslips or filters and grown to 60–70% confluence before treatment for 6 or 12 h with Lipofectamine reagent (Gibco/BRL) together with

expression plasmid, according to the manufacturer's instructions. Cells on coverslips were allowed to recover in growth medium for 24–30 h before fixation. MDCK cells were grown for 3 days to allow polarization. For antisense experiments, Cos-7 cells received 4-h treatments with 20–500 nm phosphothiolated 20mer LIS1 oligonucleotide corresponding to the N-terminal coding region (oligo2, 5′-GACCAAGAGGTCCACCTGAC) or a control oligonucleotide on 3 successive days according to the manufacturer's protocol (Oligos Etc, Wilsonville, Oregon). Daily total protein concentrations were determined by Bradford Assay; levels of LIS1 were measured from immunoblots. For phenotypic analysis and cell counts, coverslips of day-three-treated cells were fixed in methanol and analysed by immunofluoresence microscopy as described below.

Immunofluorescence microscopy.

Cells were generally fixed in methanol at –20 °C for 10 min. To visualize cortical staining, cells were preextracted in 0.5% Triton X-100 in PHEM buffer (120 mM PIPES, 50 mM HEPES, 20 mM EGTA and 4 mM magnesium acetate, pH 6.9) for 1 min, fixed in 3% paraformaldehyde for 20 min, and then in methanol at –20 °C for 6 min (Busson *et al.* 1998; Figs. 4a–f and 6). Coverslips were blocked for 30 min with 1% BSA, incubated for 1 h in primary antibody, washed, and incubated for 1 h in secondary antibodies (Alexa Fluor 488, goat anti-rabbit and mouse were from Molecular Probes; CY3 goat anti-rabbit and mouse were from Jackson Immunoresearch). To stain chromosomes, cells were subsequently exposed to Hoechst for 5 min. Coverslips were mounted with 0.1% p-phenylenediamine in PBS with 50% glycerol. A Zeiss Axiophot photomicroscope equipped for epifluoresence was used to visualize the samples. Images were obtained with an ORCA digital Camera (Hammamatsu, Japan), and recorded with Metamorph software (Universal Imaging Corp.) or with film. Confocal microscopy was carried out with a Nikon Diaphot 200 microscope using the BioRad MRC1000 system with a Kr/Ar laser. Images were overlaid and cropped in Adobe Photoshop 4.0 (Adobe Systems Inc., Mountain View, California). Corel Draw 7 (Corel Corp., Ottawa, Canada) was used to assemble figures. For Golgi localization, cells were transfected with LIS1 cDNA plus GFP-*N*-acetyl glucosamine transferase cDNA (NAGT)[17]. Endosomes and lysosomes were detected by labelling cells with tetramethylrhodamine isothiocyanate (TRITC)–transferrin[17] or Lysotracker (Molecular Probes) after 4 h of serum starvation.

Microinjection.

To visualize chromosomes, cultured NRK cells were incubated for 30 min with 1 μg ml^{-1} vital Hoechst 33258 (Sigma) and then microinjected during prophase (before nuclear-envelope breakdown). For most experiments, a purified immunoglobulin G (IgG) fraction from our anti-LIS1 peptide polyclonal antibody was used (8–20 mg ml^{-1}). Pre-immune and peptide-blocked immune IgG fractions were used as controls. Monoclonal antibody against cytoplasmic-dynein intermediate chain 70.1 was prepared as described[41]. All antibodies were dialysed overnight into microinjection buffer (50 mM potassium glutamate and 0.5 mM $MgCl_2$, pH 7.0) just before use. Imaging was carried out on an Axiovert S100 TV inverted Microscope (Zeiss) with a 40×, NA 0.75 Neofluar lens. Images were acquired at 1 frame every 2 min, except during anaphase when 1 image was taken every minute, using a NTE/CCD-512 EBFT digital camera (Roper Scientific Inc., Trenton, New Jersey), and processed using in-house custom software. Time-lapse movies were constructed using Adobe Premiere.

RECEIVED 9 JUNE 2000; REVISED 19 JULY 2000; ACCEPTED 21 AUGUST 2000;
PUBLISHED 29 SEPTEMBER 2000.

1. Dobyns, W. B., Reiner, O., Carrozzo, R. & Ledbetter, D. H. Lissencephaly. A human brain malformation associated with deletion of the LIS1 gene located at chromosome 17p13. *J. Am. Med. Soc.* **270,** 2838–2842 (1993).
2. Reiner, O. *et al.* Isolation of a Miller–Dieker lissencephaly gene containing G protein β-subunit-like repeats. *Nature* **364,** 717–721 (1993).
3. Lo Nigro, C. *et al.* Point mutations and an intragenic deletion in *LIS1*, the lissencephaly causative gene in isolated lissencephaly sequence and Miller–Dieker syndrome. *Hum. Mol. Genet.* **6,** 157–164 (1997).
4. Chong, S. S. *et al.* A revision of the lissencephaly and Miller–Dieker syndrome critical regions in chromosome 17p13.3. *Hum. Mol. Genet.* **6,** 147–155 (1997).
5. Mizuguchi, M., Takashima, S., Kakita, A., Yamada, M. & Ikeda, K. Lissencephaly gene product. Localization in the central nervous system and loss of immunoreactivity in Miller–Dieker syndrome. *Am. J. Pathol.* **147,** 1142–1151 (1995).
6. Fogli, A. *et al.* Intracellular levels of the LIS1 protein correlate with clinical and neuroradiological findings in patients with classical lissencephaly [see comments]. *Ann. Neurol.* **45,** 154–161 (1999).
7. Hirotsune, S. *et al.* Graded reduction of *Pafah1b1 (Lis1)* activity results in neuronal migration defects and early embryonic lethality. *Nature Genet.* **19,** 333–339 (1998).
8. Hattori, M., Adachi, H., Tsujimoto, M., Arai, H. & Inoue, K. Miller–Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor. *Nature* **370,** 216–218 (1994).
9. Xiang, X., Osmani, A. H., Osmani, S. A., Xin, M. & Morris, N. R. *NudF*, a nuclear migration gene in *Aspergillus nidulans*, is similar to the human *LIS-1* gene required for neuronal migration. *Mol. Biol. Cell* **6,** 297–310 (1995).
10. Plamann, M., Minke, P. F., Tinsley, J. H. & Bruno, K. S. Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. *J. Cell Biol.* **127,** 139–149 (1994).
11. Xiang, X., Beckwith, S. M. & Morris, N. R. Cytoplasmic dynein is involved in nuclear migration in *Aspergillus nidulans. Proc. Natl Acad. Sci. USA* **91,** 2100–2104 (1994).
12. Morris, N. R. Nuclear migration. From fungi to the mammalian brain. *J. Cell Biol.* **148,** 1097–1101 (2000).
13. Geiser, J. R. *et al. Saccaromyces cerevisiae* genes required in the absence of the *CIN8*-encoded spindle motor act in functionally diverse mitotic pathways. *Mol. Biol. Cell* **8,** 1035–1050 (1997).
14. Swan, A., Nguyen, T. & Suter, B. *Drosophila* Lissencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. *Nature Cell Biol.* **1,** 444–449 (1999).
15. Liu, Z., Xie, T. & Steward, R. *Lis1*, the *Drosophila* homolog of a human lissencephaly disease gene, is required for germline cell division and oocyte differentiation. *Development* **126,** 4477–4488 (1999).
16. Echeverri, C. J., Paschal, B. M., Vaughan, K. T. & Vallee, R. B. Molecular characterization of the 50kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J. Cell Biol.* **132,** 617–633 (1996).
17. Burkhardt, J. K., Echeverri, C. J., Nilsson, T. & Vallee, R. B. Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J. Cell Biol.* **139,** 469–484 (1997).
18. Wolff, A. *et al.* Distribution of glutamylated alpha and beta-tubulin in mouse tissues using a specific monoclonal antibody, GT335. *Eur. J. Cell Biol.* **59,** 425–432 (1992).
19. Bobinnec, Y. *et al.* Centriole disassembly *in vivo* and its effect on centrosome structure and function in vertebrate cells. *J. Cell Biol.* **143,** 1575–1589 (1998).
20. Carminati, J. L. & Stearns, T. Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex. *J. Cell Biol.* **138,** 629–641 (1997).
21. Skop, A. R. & White, J. G. The dynactin complex is required for cleavage plane specification in early *Caenorhabditis elegans* embryos. *Curr. Biol.* **8,** 1110–1116 (1998).
22. Gonczy, P., Pichler, S., Kirkham, M. & Hyman, A. A. Cytoplasmic dynein is required for distinct aspects of MTOC positioning, including centrosome separation, in the one cell stage *Caenorhabditis elegans* embryo. *J. Cell Biol.* **147,** 135–150 (1999).
23. Busson, S., Dujardin, D., Moreau, A., Dompierre, J. & De Mey, J. R. Dynein and dynactin are localized to astral microtubules and at cortical sites in mitotic epithelial cells. *Curr. Biol.* **8,** 541–544 (1998).
24. Reinsch, S. & Karsenti, E. Orientation of spindle axis and distribution of plasma membrane proteins during cell division in polarized MDCKII cells. *J. Cell Biol.* **126,** 1509–1526 (1994).
25. Steuer, E. R., Wordeman, L., Schroer, T. A. & Sheetz, M. P. Localization of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature* **345,** 266–268 (1990).
26. Pfarr, C. M. *et al.* Cytoplasmic dynein is localized to kinetochores during mitosis. *Nature* **345,** 263–265 (1990).
27. Rieder, C. L. & Alexander, S. P. Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J. Cell Biol.* **110,** 81–95 (1990).
28. Vallee, R. B. A taxol dependent procedure for the purification of microtubules and MAPs. *J. Cell Biol.* **92,** 435–442 (1982).
29. Sapir, T., Elbaun, M. & Reiner, O. Reduction of microtubule catastrophe events by LIS1, platelet-activating factor acetylhydrolase subunit. *EMBO J.* **16,** 6977–6984 (1997).
30. Pierre, P., Scheel, J., Rickard, J. E. & Kreis, T. E. CLIP-170 links endocytic vesicles to microtubules. *Cell* **70,** 887–900 (1992).
31. Morrison, E. E., Wardleworth, B. N., Askham, J. M., Markham, A. F. & Meredith, D. M. EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. *Oncogene* **17,** 3471–3477 (1998).
32. Perez, F., Diamantopoulos, G. S., Stalder, R. & Kreis, T. E. CLIP-170 highlights growing microtubule ends *in vivo*. *Cell* **96,** 517–527 (1999).
33. Mimori-Kiyosue, Y., Shiina, N. & Tsukita, S. The dynamic behavior of the APC-binding protein EB1 on the distal ends of microtubules. *Curr. Biol.* **10,** 865–868 (2000).
34. Vaughan, K. T., Hughes, S. H., Echeverri, C. J., Faulkner, N. F. & Vallee, R. B. Co-localization of dynactin and cytoplasmic dynein with CLIP-170 at microtubule distal ends. *J. Cell Sci.* **112,** 1437–1447 (1999).
35. Berrueta, L., Tirnauer, J. S., Schuyler, S. C., Pellman, D. & Bierer, B. E. The APC-associated protein EB1 associates with components of the dynactin complex and cytoplasmic dynein intermediate chain. *Curr. Biol.* **9,** 425–428 (1999).
36. Paschal, B. M. *et al.* Characterization of 50 kD polypeptide in cytoplasmic dynein preparations reveals a complex with p150^{Glued} and a novel actin. *J. Biol. Chem.* **268,** 15318–15323 (1993).
37. Merdes, A., Ramyar, K., Vechio, J. D. & Cleveland, D. W. A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. *Cell* **87,** 447–458 (1996).
38. Vallee, R. B. & Sheetz, M. P. Targeting of motor proteins. *Science* **271,** 1539–1544 (1996).
39. Starr, D. A., Williams, B. C., Hays, T. S. & Goldberg, M. L. ZW10 helps recruit dynactin and dynein to the kinetochore. *J. Cell Biol.* **142,** 763–774 (1998).
40. Muhua, L., Adames, N. R., Murphy, M. D., Shields, C. R. & Cooper, J. A. A cytokinesis checkpoint requiring the yeast homologue of an APC- binding protein. *Nature* **393,** 487–491 (1998).
41. O'Connell, C. B. & Wang, Y. Mammalian spindle orientation and position respond to changes in cell shape in a dynein-dependent fashion. *Mol. Biol. Cell* **11,** 1765–1774 (2000).
42. Hinchcliffe, E. H., Cassels, G. O., Rieder, C. L. & Sluder, G. The coordination of centrosome reproduction with nuclear events of the cell cycle in the sea urchin zygote. *J. Cell Biol.* **140,** 1417–1426 (1998).
43. Williams, B. C., Karr, T. L., Montgomery, J. M. & Goldberg, M. L. The *Drosophila l(1)zw10* gene product, required for accurate mitotic chromosome segregation, is redistributed at anaphase onset. *J. Cell Biol.* **118,** 759–773 (1992).
44. Vallee, R. B., Faulkner, N. E. & Tai, C. The role of cytoplasmic dynein in the human brain developmental disease lissencephaly. *Biochim. Biophys. Acta* **1496,** 89–98 (2000).
45. Chenn, A. & McConnell, S. K. Cleavage orientation and the asymmetric inheritance of notch1 immunoreactivity in mammalian neurogenesis. *Cell* **82,** 631–641 (1995).
46. McConnell, S. K. & Kaznowski, C. E. Cell cycle dependence of laminar determination in developing neocortex. *Science* **254,** 282–285 (1991).
47. Vaughan, K. T. & Vallee, R. B. Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150^{Glued}. *J. Cell Biol.* **131,** 1507–1516 (1995).
48. Garces, J. A., Clark, I. B., Meyer, D. I. & Vallee, R. B. Interaction of the p62 subunit of dynactin with Arp1 and the cortical actin cytoskeleton. *Curr. Biol.* **9,** 1497–1500 (1999).
49. Gilbert, S. *Developmental Biology*, (Sinauer, Sunderland, Massachusetts, 1994).

ACKNOWLEDGEMENTS

We thank S. Lambert and E. Hinchcliffe for critical reading of the manuscript, and G. Sluder for helpful comments. We also thank O. Reiner, K. Pfister, H. Goodson, A. Mikami, D. Meyer and P. Denoulet for antibody reagents, D. Shima and G. Warren for GFP-*N*-acetyl glucosamine transferase cDNA, and J. De Mey for the MDCK cell line. This work was supported by NIH grants GM47434 and HD61982, the March of Dimes Birth Defects Foundation, the Human Frontier Science Program, and the H. Arthur Smith Charitable Foundation.

Correspondence and requests for materials should be addressed to R.B.V. Supplementary Information is available on our website (http://www.cellbio.nature.com).