

Role of dynein, dynactin, and CLIP-170 interactions in LIS1 kinetochore function

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Mutations in the human LIS1 gene cause type I lissencephaly, a severe brain developmental disease involving gross disorganization of cortical neurons. In lower eukaryotes, LIS1 participates in cytoplasmic dynein-mediated nuclear migration. We previously reported that mammalian LIS1 functions in cell division and coimmunoprecipitates with cytoplasmic dynein and dynactin. We also localized LIS1 to the cell cortex and kinetochores of mitotic cells, known sites of dynein action. We now find that the COOH-terminal WD repeat region of LIS1 is sufficient for kinetochore targeting. Overexpression of this domain or full-length LIS1 displaces CLIP-170 from this site without affecting dynein and other kinetochore markers. The NH₂-terminal self-association domain of LIS1 displaces endogenous LIS1 from the kinetochore, with no effect on

CLIP-170, dynein, and dynactin. Displacement of the latter proteins by dynamitin overexpression, however, removes LIS1, suggesting that LIS1 binds to the kinetochore through the motor protein complexes and may interact with them directly. We find that of 12 distinct dynein and dynactin subunits, the dynein heavy and intermediate chains, as well as dynamitin, interact with the WD repeat region of LIS1 in coexpression/coimmunoprecipitation and two-hybrid assays. Within the heavy chain, interactions are with the first AAA repeat, a site strongly implicated in motor function, and the NH₂-terminal cargo-binding region. Together, our data suggest a novel role for LIS1 in mediating CLIP-170–dynein interactions and in coordinating dynein cargo-binding and motor activities.

Introduction

Mutations in the human LIS1 gene are responsible for the autosomal dominant brain developmental disease, type I lissencephaly (Reiner et al., 1993). This condition involves gross disorganization of neurons within the cerebral cortex, and is thought to reflect a failure in normal neuronal migration during early development.

Genetic studies in lower eukaryotes have indicated that LIS1 homologues participate in dynein-mediated nuclear migration (Xiang et al., 1994; Geiser et al., 1997). In verte-

brates, LIS1 was initially identified as a noncatalytic subunit of brain platelet-activating factor acetylhydrolase (PAFAH)* (Hattori et al., 1993). We, and others, have more recently found LIS1 to interact biochemically with cytoplasmic dynein and dynactin (Faulkner et al., 2000; Sasaki et al., 2000; Smith et al., 2000). We also obtained evidence for a role for LIS1 in vertebrate cell division, raising the possibility that the errors in neuronal distribution characteristic of lissencephaly arise from defects in the genesis of neuronal progenitors (Faulkner et al., 2000). Overexpression of LIS1, application of LIS1 antisense oligonucleotides, and microinjection of anti-LIS1 antibody all produced potent mitotic defects, including spindle misorientation and chromosome misalignment at the metaphase plate. Consistent with a mitotic function, LIS1 localized prominently to prometaphase kinetochores and to the cell cortex of dividing vertebrate cultured cells, known binding sites for cytoplasmic dynein and its accessory complex, dynactin.

The molecular relationship between dynein, dynactin, and LIS1 is poorly understood. Dynein and dynactin are each large, multisubunit complexes. Dynein consists of a heavy chain (HC), responsible for ATPase activity and force production, plus a variety of accessory subunits (intermediate,

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*Abbreviations used in this paper: aa, amino acid(s); HA, hemagglutinin; HC, heavy chain; IC, intermediate chain; LC, light chain; LIC, light intermediate chain; LIS1 N, NH₂-terminal LIS1 fragment; PAFAH, platelet-activating factor acetylhydrolase.

Key words: dynein; dynactin; LIS1; CLIP-170; kinetochores

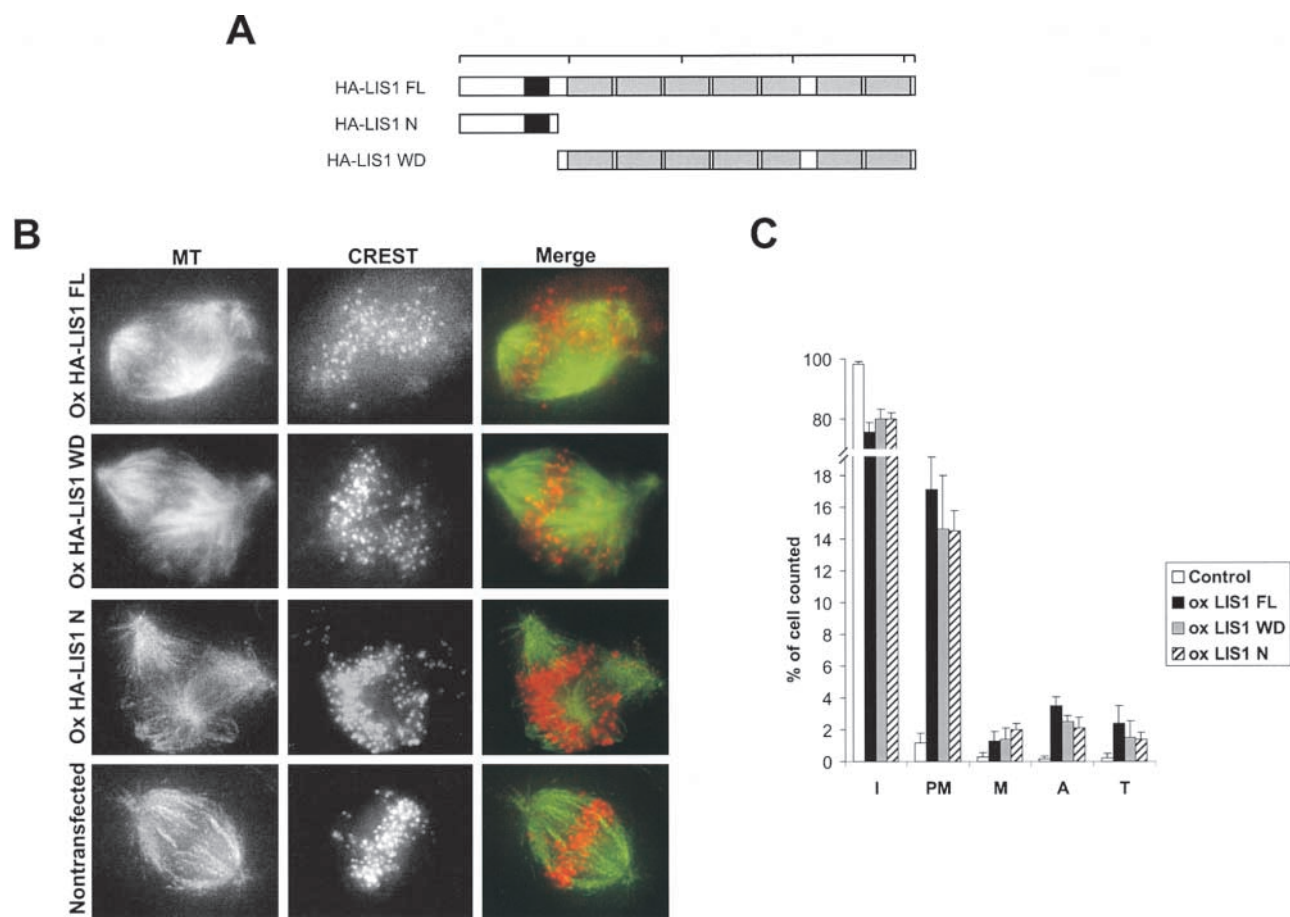


Figure 1. **Phenotypic analysis of LIS1 fragments.** (A) Schematic diagram of LIS1 domains, showing a predicted coiled-coil region (filled box) from aa 51 to 78 and a series of seven WD repeats (shaded boxes) between aa 97 and 408. (B) Effects of overexpression of LIS1 fragments on mitotic spindle morphology. Spindle defects (untransfected, 4%; LIS1 FL, 36%; LIS1 WD, 65.4%; LIS1N, 61.2%) included multiple spindle poles, detached poles, and unfocused poles. (C) Perturbation of mitosis by LIS1 fragment overexpression. Cell cycle stage was scored for nontransfected control cells (control), cells overexpressing full-length LIS1 (Ox LIS1 FL), and the NH₂- and COOH-terminal fragments (Ox LIS1 N and Ox LIS1 WD). Full-length LIS1 and NH₂- and COOH-terminal fragments all produced similar pronounced increases in mitotic index. Values are means \pm SD from four independent experiments with \sim 2,000 total cells counted in each case. A, anaphase; I, interphase; M, metaphase; PM, prometaphase; T, telophase.

light intermediate, and light chains; ICs, LICs, and LCs) implicated in binding to diverse forms of subcellular cargo (Vaughan and Vallee, 1995; Tynan et al., 2000b; Ye et al., 2000; Tai et al., 2001). Dynactin consists of a short filament of the actin-related protein Arp1 and a variety of associated polypeptides. Dynactin has been found to link dynein to the prometaphase kinetochore (Echeverri et al., 1996) and the surface of Golgi membranes (Burkhardt et al., 1997; Roghi and Allan, 1999), suggesting that it participates in dynein cargo binding. Dynactin has also been reported to stimulate dynein processivity (King and Schroer, 2000). The ICs of cytoplasmic dynein interact directly with the p150^{Glued} subunit of dynactin in *in vitro* assays (Karki and Holzbaaur, 1995; Vaughan and Vallee, 1995). However, the interaction between the two intact complexes has been observed only at very limited levels (Kini and Collins, 2001), suggesting that it is weak or regulated (Vaughan et al., 2001). LIS1 has been reported to interact with a portion of the cytoplasmic dynein HC in yeast two-hybrid and coexpression/coimmunoprecipitation assays (Sasaki et al., 2000). Whether it interacts with other subunits of dynein, whether it interacts with dynactin

independently, and how these interactions relate to dynein function and subcellular organization are poorly understood.

How the structural organization of LIS1 relates to its function is also poorly understood. LIS1 is predicted to consist of two distinct domains. The NH₂-terminal 97 amino acids (aa) contains a short region of predicted coiled-coil that has been implicated in self-association (Ahn and Morris, 2001). The COOH-terminal 317 aa contains a series of seven WD repeats, which in the β subunit of the heterotrimeric G proteins and other polypeptides constitute a β -propeller fold (Wall et al., 1995). COOH-terminal deletions of LIS1 are sufficient to cause lissencephaly (Cardoso et al., 2000), indicating that the WD repeat domain is important functionally. This region has been implicated in binding to the catalytic subunit of PAFAH (Kitagawa et al., 2000; Niethammer et al., 2000; Sweeney et al., 2000). Whether this is its sole function, or whether it also functions in the dynein pathway is unknown. LIS1 has been reported to interact with two additional classes of protein in the dynein pathway, NudC and NudE/NudEL (Morris et al., 1998; Efimov and Morris, 2000; Feng et al., 2000; Niethammer et al., 2000),

but their role in LIS1 function and their binding sites within LIS1 are uncertain.

We report here that LIS1 associates with kinetochores through its WD repeat domain. Using dominant inhibitory dynein and LIS1 constructs, we find LIS1 to be linked to the kinetochore through the motor protein complexes, rather than the reverse. We find that the WD repeat domain of LIS1 interacts with three distinct subunits of the dynein and dynactin complexes, representing both motor and cargo-binding regions. Overexpression of full-length LIS1 and its WD repeat fragment specifically displaced CLIP-170 from mitotic kinetochores. Together, these results suggest a direct role for LIS1 in novel forms of dynein/dynactin regulation.

Results

Phenotypic effects of LIS1 fragments

Previous work from this lab implicated LIS1 in mitotic chromosome alignment and spindle orientation (Faulkner et al., 2000). To determine which portion of the LIS1 molecule is involved in these activities, we constructed two cDNAs encoding HA-tagged fragments of LIS1 corresponding to the NH₂-terminal 87 aa, comparable to the 89-aa product resulting from a known disease-causing deletion (Cardoso et al., 2000), and the COOH-terminal 327 aa, respectively (Fig. 1 A). The NH₂-terminal fragment contains a 28-aa predicted coiled-coil domain, whereas the COOH-terminal fragment contains seven WD repeat motifs. To our surprise, overexpression of each fragment produced a dramatic increase in mitotic index comparable to that observed with full-length LIS1 (Fig. 1, B and C), with a greater number of multipolar spindles and a more dramatic disruption of spindle organization (Fig. 1 B). The more severe phenotype is reminiscent of that produced by overexpression of the dynamitin subunit of dynactin (Echeverri et al., 1996). However, as reported previously for full-length LIS1 (Faulkner et al., 2000) and unlike dynamitin, no effect on Golgi morphology was detectable with the LIS1 fragments (unpublished data).

To gain insight into the manner in which the individual fragments produced these effects, we examined their ability to associate with prometaphase kinetochores, a site at which we had previously observed endogenous LIS1 using immunofluorescence microscopy (Faulkner et al., 2000). We also treated cells with nocodazole, which allows outer kinetochore components to accumulate above their normal levels (Echeverri et al., 1996; King et al., 2000; Hoffman et al., 2001). Full-length HA-tagged LIS1 exhibited dramatic localization to prometaphase kinetochores, identified by costaining with CREST human autoimmune antiserum in both COS-7 (Fig. 2 A, upper row) and HeLa cells (unpublished data), adding strong support to the identification of LIS1 as a kinetochore component. Within the kinetochore, LIS1 staining was slightly peripheral to the CREST signal, consistent with outer kinetochore localization. In cells exposed to nocodazole for prolonged periods (see below), LIS1 exhibited an elongated crescent shape, as observed for dynein and other outer kinetochore components (Echeverri et al., 1996; King et al., 2000; Hoffman et al., 2001). Clear kinetochore localization was also observed with the WD repeat fragment

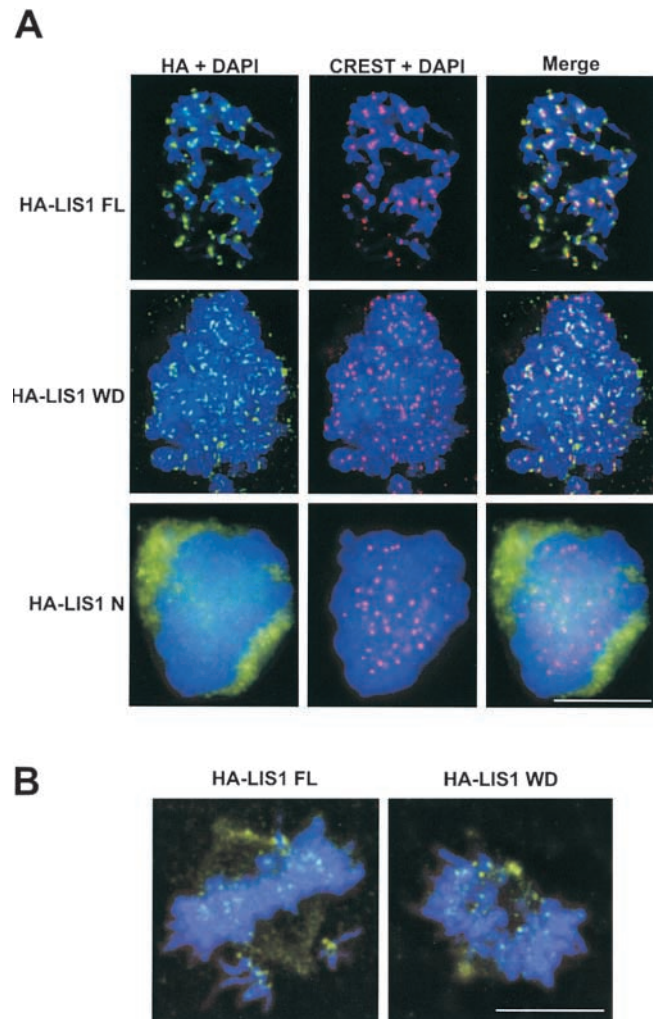


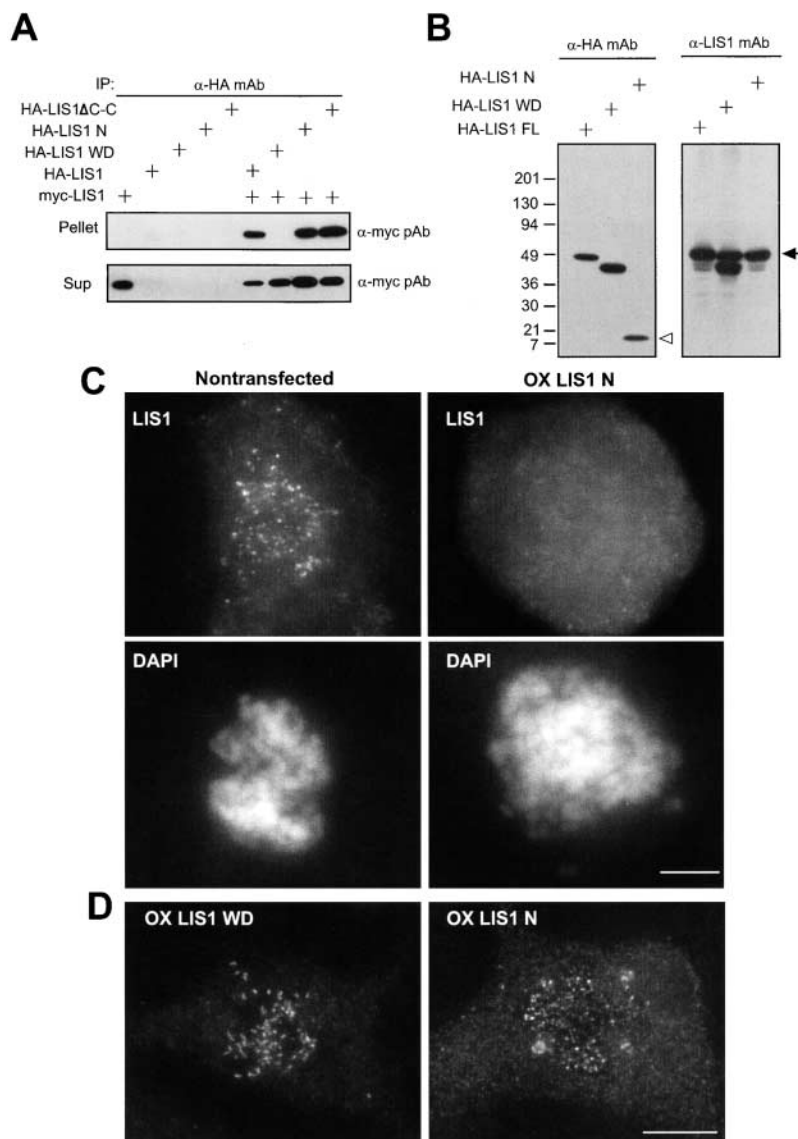
Figure 2. **Kinetochore localization of LIS1 fragments.** (A) Transfected COS-7 cells were treated with nocodazole before fixation for immunofluorescence. Cells were triple-labeled for LIS1 and its fragments using anti-HA monoclonal antibody (green), inner kinetochore antigens using CREST human autoimmune antiserum (red), and DNA using DAPI (blue). (B) Kinetochore localization in untreated cells. Merged images of DNA (blue) and anti-HA antibody-labeled LIS1 signals (green) in COS-7 cells transfected with either HA-LIS1 FL or HA-LIS1 WD constructs. Bars, 5 μ m.

of LIS1 alone. This effect was more pronounced in nocodazole-treated cells (Fig. 2 A, middle row), but was also readily observed in untreated cells (Fig. 2 B). In contrast, specific localization of the NH₂-terminal LIS1 fragment (LIS1 N) was not generally observed (Fig. 2 A, lower row), though very weak kinetochore staining could be detected in occasional cells (unpublished data). Together, these results indicate that the WD repeat domain of LIS1 is sufficient for kinetochore targeting.

Hierarchy of LIS1 interactions at the kinetochore

Despite this difference in kinetochore binding behavior, the two fragments produce phenotypes of comparable severity (see above). The NH₂ terminus of the LIS1-related *Aspergillus* protein NudF has been implicated in self-association (Ahn and Morris, 2001), raising the possibility that the corresponding portion of LIS1 might interact with the endoge-

Figure 3. Effects of LIS1 fragments on kinetochore composition. (A) Self-association of LIS1 N. Myc-tagged LIS1 full-length cDNA was coexpressed with different HA-tagged LIS1 fragments (Fig. 1) and an HA-tagged LIS1 coiled-coil deletion construct (HA-LIS1 Δ C-C), and anti-HA immunoprecipitates were immunoblotted with anti-myc antibody. Clear coimmunoprecipitation was observed only between constructs including the NH₂-terminal region of LIS1. (B) Specificity of LIS1 monoclonal antibody for WD repeat region. COS-7 cells were transfected with HA-tagged LIS1 constructs. Cell lysates were loaded into 5–15% gradient gels and analyzed by immunoblotting with anti-HA monoclonal antibody, which recognized all LIS1 constructs, and monoclonal anti-LIS1 (Sapir et al., 1997; Faulkner et al., 2000). The latter recognized endogenous and full-length HA-LIS1 (arrow), as well as the slightly smaller WD repeat fragment, but not the 10-kD LIS1 N (open arrow-head). (C) Effect of LIS1 N on endogenous LIS1 localization. In contrast to nontransfected cells, endogenous LIS1 protein detected with the LIS1 monoclonal antibody was displaced from kinetochores by LIS1 N overexpression. (D) Effect of LIS1 N and LIS1 WD on endogenous dynein IC localization. Dynein staining at the kinetochore persisted in cells overexpressing either fragment. Bars, 5 μ m.



nous full-length protein and interfere with its behavior. In coexpression experiments, we observed the NH₂-terminal fragment coimmunoprecipitating with itself and full-length LIS1, but not with the WD region (Fig. 3 A). In contrast to previous results with *NudF*, removal of the predicted coiled-coil (HA-LIS1 Δ C-C) had no effect on self-association, suggesting that the entire NH₂-terminal domain participates in this activity. To determine whether the NH₂-terminal domain can displace the endogenous wild-type protein from kinetochores, we examined cells overexpressing the NH₂-terminal construct for endogenous LIS1 staining. For this purpose, we used a monoclonal antibody (Sapir et al., 1997) that we determined to react with the WD repeat region of LIS1 (Fig. 3 B). In high expressers, the endogenous LIS1 kinetochore staining was, indeed, dramatically reduced (Fig. 3 C, right column).

We previously reported that dynein and dynactin remain at the prometaphase kinetochore in LIS1-overexpressing cells (Faulkner et al., 2000), though our current data (Fig. 2) indicate that the full-length epitope-tagged LIS1 protein occupies the kinetochore under these conditions. To test the effects of removing endogenous LIS1 from this site, we over-

expressed LIS1 N. No effect on the dynein (Fig. 3 D) and dynactin (unpublished data) signals was observed in cells overexpressing the LIS1 fragment versus untransfected cells (unpublished data).

To test whether LIS1 displaced other kinetochore proteins, we stained cells overexpressing full-length LIS1 and the NH₂-terminal and WD repeat fragments with antibodies against CENP-E, CENP-F, Bub1, BubR1, Mad2, and CLIP-170. No effect was observed, except in the case of CLIP-170, which was completely displaced by the full-length and WD repeat LIS1 constructs (Fig. 4; unpublished data).

To test whether LIS1 requires dynein/dynactin for kinetochore binding, we overexpressed the dynamitin subunit of dynactin, which dissociates dynactin into smaller subcomplexes and releases dynein from the kinetochore (Echeverri et al., 1996). We now find that epitope-tagged dynamitin persists at the kinetochore under these conditions (Fig. 5 A). This behavior suggests an additional effect of dynamitin overexpression: competition for binding to the dynein/dynactin anchoring protein ZW10 (Starr et al., 1998). As expected, Arp1, a dynactin subunit, was displaced from the ki-

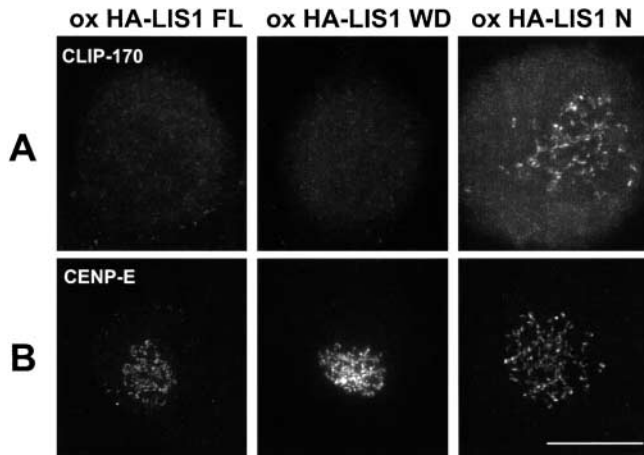


Figure 4. **Displacement of CLIP-170 by overexpression of LIS1 fragments.** HeLa cells were transfected with LIS1 constructs and treated with 10 μ M nocodazole for 1 h before fixation for immunofluorescence. (A) CLIP-170 staining in LIS1 overexpressors. Both LIS1 FL and WD constructs clearly displaced CLIP-170 from kinetochores, whereas LIS1 N had no effect. (B) CENP-E staining in LIS1 overexpressors. CENP-E kinetochore signal was unaffected by overexpression of any of the LIS1 constructs. Bar, 10 μ m.

netochore (Fig. 5 B, upper left). The LIS1 signal was also greatly diminished (Fig. 5 B, upper right). Together, our data indicate that the localization of LIS1 to the kinetochore requires dynein and dynactin rather than the reverse.

LIS1 interacts through its WD repeat domain with multiple sites in dynein and dynactin

The dependence of LIS1 kinetochore targeting on dynein and dynactin supports a physiological interaction with the two complexes. To define the molecular basis for these interactions further, we explored whether LIS1 can interact with subunits in each complex. LIS1 was coexpressed with cytoplasmic dynein and dynactin subunits, and interactions were assessed by coimmunoprecipitation.

Both the HCs and ICs of cytoplasmic dynein coimmunoprecipitated with LIS1, as did the dynamitin subunit of dynactin (Fig. 6 A). LIC1 and LIC2 and LCs (Tctex-1, RP3, and LC8) of the dynein complex, and the p150^{Glued}, Arp1, and p62 subunits of the dynactin complex were all negative in this assay. These results indicate that LIS1 interacts with each complex independently and, in the case of cytoplasmic dynein, through multiple subunit interactions. We also examined these interactions by immunoprecipitation from [³⁵S]methionine-labeled total cell extracts. In each case (HC, IC2C, and dynamitin), LIS1 was the major nondynein/dynactin polypeptide pulled down, with minor background bands also visible (unpublished data).

We also used the yeast two-hybrid assay as a further test for interaction specificity. A positive reaction was observed between LIS1 and the cytoplasmic dynein HCs and ICs and dynamitin (Fig. 6 B). In the case of the HC, three fragments were analyzed for interactions. HC N649C907 and HC N1874C2124 showed positive reactions, whereas HC N547C649 did not (see next section for further detail). Thus, together our data support a direct interaction

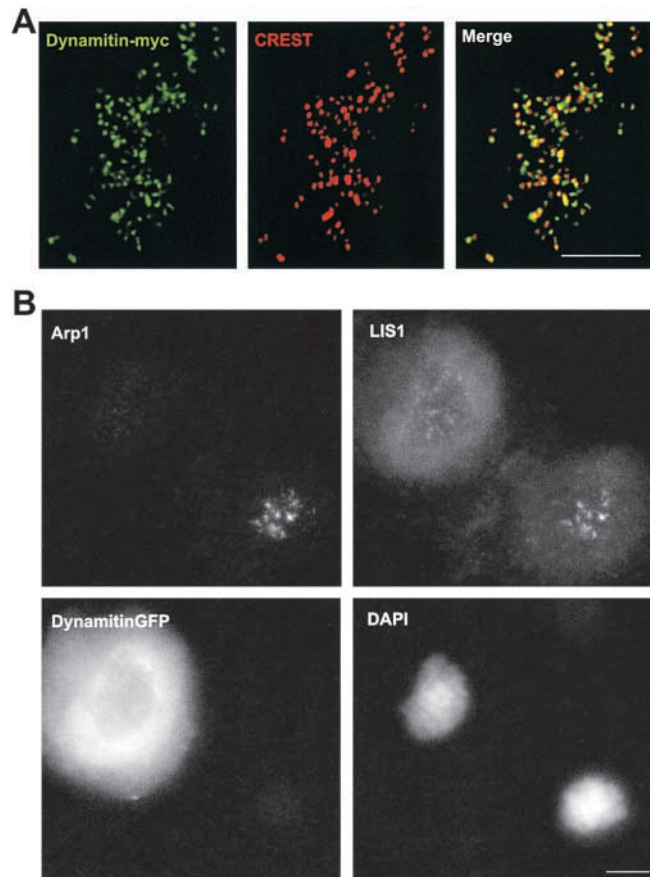


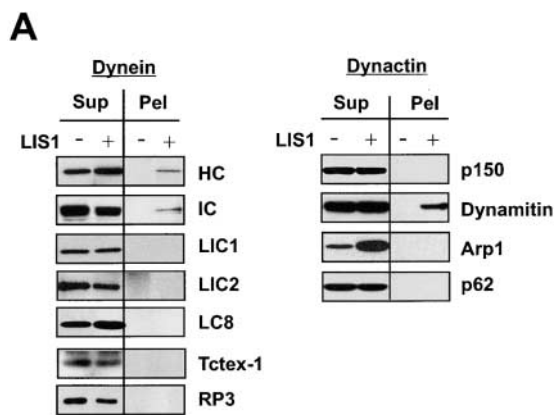
Figure 5. **Displacement of LIS1 by dynamitin overexpression.** (A) Confocal images of COS-7 cells transfected with dynamitin-myc and stained with polyclonal anti-myc antibody (green) and CREST human auto-antiserum (red). In addition to its disruptive effect on the dynactin complex, overexpressed dynamitin associates with kinetochores. (B) Nocodazole-treated COS-7 cells transfected with dynamitin-green fluorescence protein (GFP) and stained with anti-Arp1, anti-LIS1, and DAPI. Arp1 and LIS1 were clearly reduced. Bars, 10 μ m.

between LIS1 and each of three distinct dynein and dynactin subunits.

To gain further insight into the molecular basis for the observed interactions, we investigated which region of LIS1 is involved. No interaction was detected using LIS1 N. Instead, each of the LIS1-interacting subunits coimmunoprecipitated with the WD repeat domain of LIS1 alone (Fig. 7). These results are consistent with a role for the WD repeat domain in linking LIS1 to the kinetochore through dynein and dynactin (see above).

Two distinct sites of the HC interact with LIS1

The dynein HC consists of two major functional regions. The NH₂-terminal aa 1100–1300 are thought to constitute the “stem” of the molecule, and contain the known sites for interaction with the cargo-binding ICs, LICs, and LCs (Habura et al., 1999; Tynan et al., 2000a; Mok et al., 2001). The remainder of the molecule represents the motor domain. The site for microtubule binding is located at the tip of a projecting stalk (aa 3160–3506; Gee et al., 1997), which resides within a series of recently predicted AAA ATPase domains (Neuwald et al., 1999). Photocleavage (Gibbons



B

Two-hybrid prey clones	Two-hybrid bait clones	
	LIS1 FL	LIS1 WD
Dynamitin	++	++
IC	+	+
HC N547C649	-	-
HC N748C907	+	+
HC N1874C2124	+	+

Figure 6. Interaction of LIS1 with multiple dynein and dynactin subunits. (A) Coimmunoprecipitation of LIS1 with dynein and dynactin subunits. Full-length HA- or myc-LIS1 was cotransfected with HC-FLAG, IC2C-myc, LIC2-FLAG, LC8-VSVG, Tctex-1-HA, RP3-HA, p150^{Glued}, dynamitin, Arp1, and p62-myc. Immunoprecipitation was performed with anti-HA or anti-myc antibody and blotted with antibodies against the epitope tag of individual dynein subunit or anti-p150^{Glued}, -dynamitin, or -Arp1. Interactions were observed with the dynein HC and IC and dynamitin. IC1A was also positive in this assay (unpublished data). (B) Two-hybrid assay of LIS1 with dynamitin, HC, and IC. LIS1 fragments were cloned into the LexA-based bait vector and full-length dynamitin, IC2C, HC N547C649, HC N748C907, and HC N1874C2124 fragments (see Fig. 8) were cloned into the prey vector. Dynamitin, IC, HC N748C907, and HC N1874C2124 were all positive in this assay. The negative HC fragment (HC N547C649) was also negative in the coimmunoprecipitation assay (see Fig. 8).

and Gibbons, 1987) or mutation (Gee et al., 1997) of the P-loop of the first AAA domain abolishes ATPase activity, implicating this site most clearly in ATP hydrolysis and force production.

The identity of the site within the HC with which LIS1 interacts should provide important insight into LIS1 function. To investigate this issue systematically, we coexpressed LIS1 with a series of motor domain and stem fragments and subfragments (Fig. 8). Surprisingly, clear binding was observed with HC fragments corresponding to each major domain (e.g., N1137flag corresponding to the entire motor domain and C1140myc corresponding to the stem domain). Analysis of subfragments of the motor domain indicated that one LIS1 binding site is located toward the NH₂ terminus of this region. During the course of this study a LIS1-

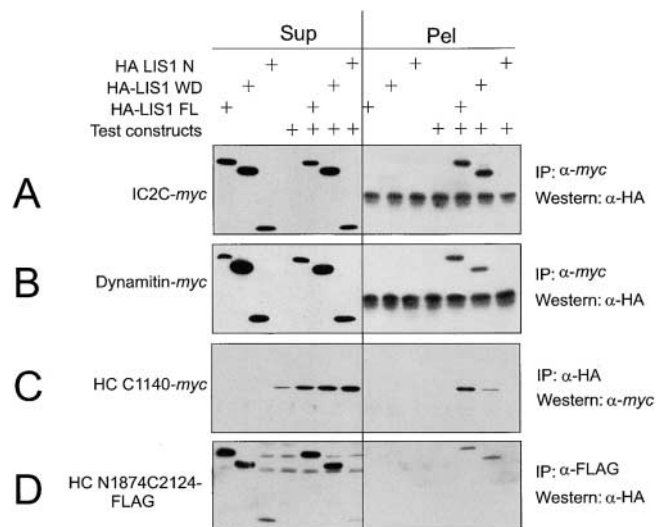


Figure 7. WD repeat domain of LIS1 mediates dynein and dynactin subunit interactions. COS-7 cells were singly- or doubly-transfected with HA-LIS1 FL, HA-LIS1 WD, HA-LIS1 N versus (A) IC2C-myc, (B) dynamitin-myc, (C) HC C1140-myc, and (D) HC N1874C2124-FLAG constructs. Immunoprecipitations were performed by using (A and B) anti-myc antibody, (C) anti-HA antibody, or (D) FLAG M2 beads. The immunoprecipitates (right) were immunoblotted with either anti-myc antibody (C) or anti-HA antibody (A, B, and D). Supernatants (left) were used to monitor protein expression. Each dynein and dynactin subunit immunoprecipitated with HA-LIS1 FL and HA-LIS1 WD, but not with HA-LIS1 N.

binding fragment from the same region encompassing part, but not all, of the first two AAA domains was also identified in a yeast two-hybrid screen (Sasaki et al., 2000). Recently, the same fragment was also found to interact with the *Aspergillus* LIS1 homologue NUDF (Hoffmann et al., 2001). To determine whether the LIS1 binding site corresponded to a predicted HC functional domain, we designed a fragment (N1874C2124flag) corresponding to the boundaries of the first AAA repeat (Neuwald et al., 1999). Clear binding with LIS1 was observed. To determine whether LIS1 binding is affected by ATP binding state, full-length HC and AAA domain constructs containing K to E mutations in the first P-loop element were tested (Fig. 8, A and B, K1910Eflag and N1874C2124^{K1910E}flag). No effect on LIS1 binding was detected, indicating that the LIS1-HC interaction does not require a functional ATPase site.

LIS1 also bound to a single discrete site within the stem region close to the binding sites for the other dynein accessory subunits (Fig. 8, A and C, and Fig. 6 B). This site is independent of that for IC binding and overlaps with that for LIC binding. To test whether LIS1 competes with the LICs for HC binding, we immunoprecipitated the LICs. LIS1 was present in the precipitates (unpublished data), indicating that LIS1 and the LICs can coexist within the same dynein complex.

Discussion

Distinct roles for LIS1 domains

As for full-length LIS1, overexpression of the LIS1 N and WD repeat domains produced pronounced increases in mitotic in-

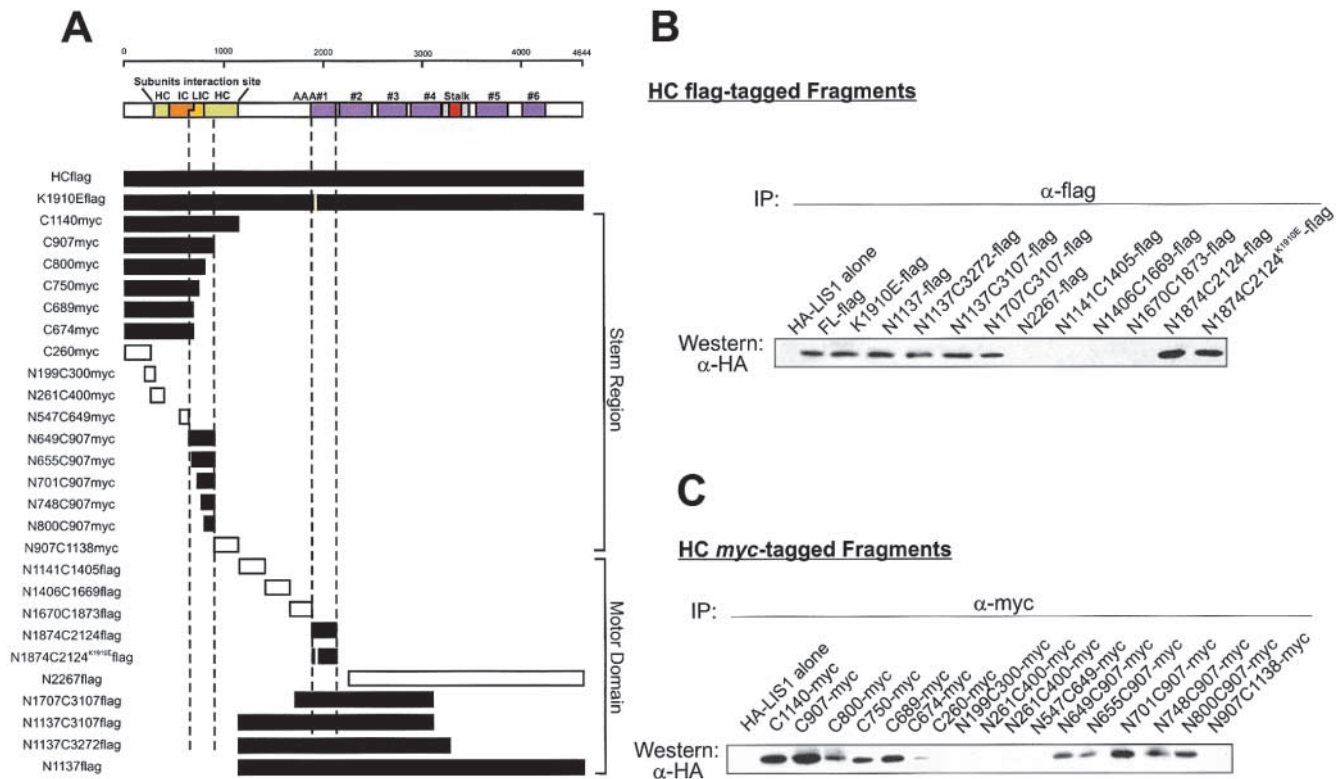


Figure 8. Two distinct sites of HC interact with LIS1. The HA-LIS1 FL construct was cotransfected with myc-tagged dynein stem region and FLAG-tagged motor domain constructs. HC fragment immunoprecipitates were immunoblotted for HA-LIS1. (A) Diagrammatic representation of dynein HC at top, showing IC- (orange), LIC- (yellow), and HC-HC (green) interaction sites (Tynan et al., 2000a), as well as the microtubule-binding stalk (gray, coiled-coil; red, microtubule-binding site) (Gee et al., 1997) and AAA domains (blue) (Neuvald et al., 1999). Filled bars represent positive interactions with LIS1 and empty bars represent negative interactions. White line in constructs represents K to E mutation at aa 1910. Dotted lines and correspond to aa 649–907 and aa 1874–2124. (B and C) Anti-HA immunoblots of HC fragment immunoprecipitates showing coprecipitating LIS1.

dex. Neither fragment affected Golgi organization (compare with Smith et al., 2000), again indicating a role in a restricted range of dynein functions (Faulkner et al., 2000). The NH₂-terminal region is involved in self-association (Fig. 3 A; Ahn and Morris, 2001), but, so far, not in interactions with dynein, dynactin (Fig. 7), α -PAFAH, or NudEL (Kitagawa et al., 2000; Niethammer et al., 2000; Sweeney et al., 2000). The NH₂-terminal domain failed to bind to kinetochores, but displaced endogenous full-length LIS1 (Fig. 3). We suggest that this behavior is likely to reflect a weakened affinity of the of LIS1 N/full-length LIS1 heterodimer for kinetochores. The resulting loss of endogenous LIS1 from this and, potentially, other sites seems likely to contribute to the mitotic phenotype.

In contrast, the WD repeat fragment showed no evidence of self-association, mediated all of the heterologous LIS1 interactions identified in this study, and targeted to kinetochores very efficiently (Fig. 2 A). The mechanism by which this region affects mitosis may involve competition with full-length LIS1 for dynein and dynactin binding, and displacement of CLIP-170 from the kinetochore (see below). Although α -PAFAH interacts with the WD repeat region of LIS1 (Kitagawa et al., 2000; Niethammer et al., 2000; Sweeney et al., 2000), it was undetectable in our cell lysates (unpublished data) and fails to associate with dynein (Niethammer et al., 2000; unpublished data). It cannot, therefore, account for the mitotic effects we observed. The precise sites of LIS1 interaction with NudE,

NudEL, and NudC are not known, nor has their fate been determined in LIS1-overexpressing cells.

Role of LIS1 in kinetochore organization and function

As in the case of full-length LIS1 (Faulkner et al., 2000), the fragments failed to displace dynein and dynactin from the kinetochore despite the efficient displacement of endogenous LIS1 by the NH₂-terminal fragment. The latter result argues strongly against a role for LIS1 in anchoring dynein and dynactin at the kinetochore. Conversely, LIS1 was displaced by dynamitin overexpression, which removes dynein and dynactin from the kinetochore. Together, our results are most clearly consistent with a model in which LIS1 is linked to the kinetochore through either dynein, dynactin, or both (Fig. 9 A).

CLIP-170 was displaced from kinetochores by overexpression of LIS1 or its WD domain (Fig. 4). Because CLIP-170 can also be displaced by dynamitin overexpression, it is likely to associate with the kinetochore indirectly through dynein or dynactin (Dujardin et al., 1998). LIS1 overexpression appears to interfere with the latter link (Fig. 4), suggesting that LIS1 may mediate the interaction between CLIP-170 and the motor protein complexes. Although this is an appealing model, it is disputed by the effects of LIS1 N overexpression, which displaces endogenous LIS1 from the kinetochore (Fig. 3 C) without affecting CLIP-170 (Fig. 4).

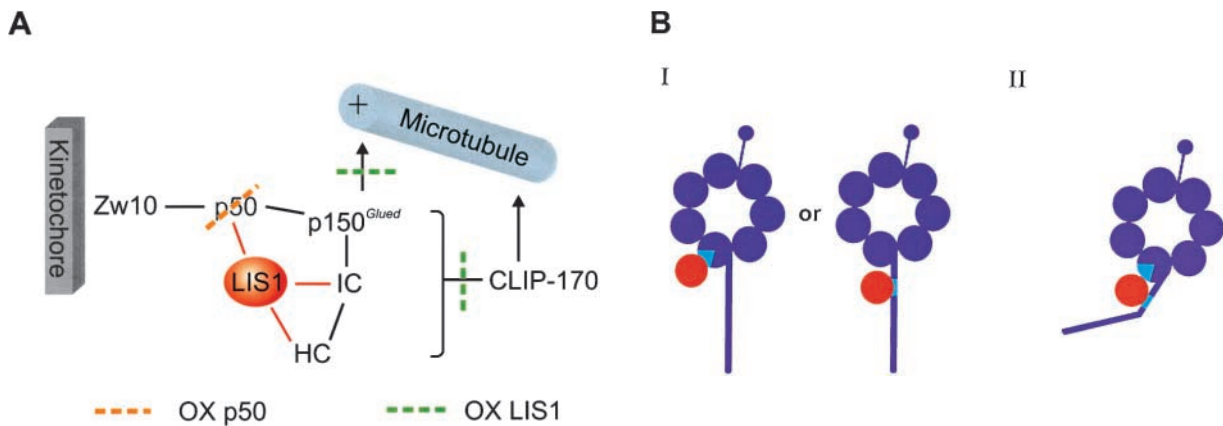


Figure 9. Schematic representations of LIS1 interactions. (A) Effect of LIS1 overexpression at kinetochores and microtubule plus ends (see text for detailed discussion and references). For clarity, dynein is represented only by its dynamitin (p50) and p150^{Glued} subunits, and dynein only by its HCs and ICs, and the interaction of the HC with microtubules is omitted. CLIP-170 associates with the kinetochore through dynein and dynactin, but the specific link is unknown. LIS1 is found in the current study to interact with p50, HC, and IC. Overexpression of dynamitin (dotted orange line) removes all polypeptides depicted downstream. Overexpression of either full-length LIS1 or LIS1 WD (dotted green line) has no effect on the association of dynein and dynactin with the kinetochore, but dissociates CLIP-170 from this site and interferes with the interaction of p150^{Glued} with microtubules (Faulkner et al., 2000). Further potential direct effects of overexpressed LIS1 or LIS1 fragments on dynein and dynactin subunits are discussed in the text. (B) Interaction of LIS1 with two dynein HC sites. (I) LIS1 is shown interacting independently with the cargo-binding dynein stem domain or the first AAA unit within the motor domain. (II) The proximity of these two sites within the folded dynein molecule may allow LIS1 to interact with both simultaneously.

All three LIS1 constructs produce more severe accumulation of cells in mitosis and more dramatic alterations in spindle morphology than are generated by a dominant-negative CLIP-170 fragment (Dujardin et al., 1998), suggesting a greater overlap in LIS1 and dynein functions. Loss of CLIP-170 almost certainly contributes to the LIS1 or LIS1 WD overexpression phenotypes, but is unlikely to explain fully the greater range of LIS1 phenotypic defects. It is uncertain whether this difference reflects a role for LIS1 in a wider range of mitotic dynein functions than CLIP-170. Our evidence for an interaction between LIS1 and subunits of dynein and dynactin suggests a direct form of regulation in which CLIP-170 is unlikely to participate. In this regard, we have already found that LIS1 displaces the p150^{Glued} subunit of dynactin from growing microtubule ends (Fig. 9 A; Faulkner et al., 2000), an effect that may weaken the interaction between kinetochores and microtubules during mitosis. It remains to be seen whether displacement of CLIP-170 from the kinetochore, which has similar microtubule-binding properties to those of p150^{Glued}, has redundant or additive effects (Fig. 9 A).

LIS1 interactions with dynein and dynactin

We have found LIS1 to interact with three different polypeptides, two in the dynein complex and a third in the dynactin complex. The ability of LIS1 to interact with subunits of both cytoplasmic dynein and dynactin suggests an important new role in linking the two complexes together. Despite extensive genetic and biochemical evidence for an interaction between the dynein IC and p150^{Glued} subunits (Karki and Holzbaur, 1995; McGrail et al., 1995; Vaughan and Vallee, 1995), only limited evidence for the copurification of the complexes has been obtained (Paschal et al., 1993; Kini and Collins, 2001). Phosphorylation of the ICs has recently been reported to regulate this interaction

(Vaughan et al., 2001), and the current study suggests that LIS1 acts as a protein cofactor that may also contribute to the stability of the cocomplex.

The association of LIS1 with the first AAA domain of the dynein HC strongly predicts an additional role in regulating motor activity. Although the mechanism by which the multiple dynein AAA domains coordinate their behavior to produce force and transmit it through the stalk is not understood, the importance of the first AAA domain has been clearly demonstrated (Gibbons and Gibbons, 1987; Gee et al., 1997).

Thus, LIS1 interacts with both cargo-binding and force-producing regions of dynein itself, as well as with dynactin, suggesting a role for LIS1 in complex and novel forms of regulation (Fig. 9 B). Conceivably, LIS1 might serve to signal whether the dynein complex is associated with cargo (such as the mitotic kinetochore) and regulate motor activity accordingly. Alternatively, it could serve to stiffen the linkage between the stem and motor domains of dynein via a mechanism reminiscent of the role of LCs in stabilizing the myosin neck region (Lowey et al., 1993). Further study of the functional properties of LIS1 is likely to provide important new insight not only into mechanisms of brain development, but into dynein regulation and function as well.

Materials and methods

Mammalian expression constructs

Most of the HC tagged constructs are described elsewhere (Mazumdar et al., 1996; Gee et al., 1997), except N907C1138-myc, N1141C1405-flag, N1406C1669-flag, N1670C1873-flag, N1874C2124-flag, and N1874C2124^{K1910E}-flag constructs, which were made by adding a NotI site, Kozak sequence, myc tag, or flag tag at the appropriate location by PCR, using VENT DNA polymerase (New England Biolabs, Inc.). They were then cloned into the NotI site of pCMV β (CLONTECH Laboratories, Inc.). LIS1 fragments were created by applying the same strategy with a hemagglutinin (HA) tag added at the NH₂ terminus of each fragment. The

NH₂-terminal 87 aa of LIS1 was used as the NH₂-terminal construct, aa 88–414 was used as the WD repeat domain construct, and an internal deletion of aa 56–78 of LIS1 was used as the coiled-coil deletion construct. Dynein IC and LIC constructs were described previously (Vaughan and Vallee, 1995; Tynan et al., 2000b), and LC constructs were obtained from Stephen King (University of Connecticut Health Center, Farmington, CT). Untagged p150^{Glued} (Vaughan and Vallee, 1995), dynamitin (Echeverri et al., 1996), Arp1 (Clark and Meyer, 1999), and p62-myc (Garces et al., 1999) were described elsewhere. All PCR-amplified constructs were sequence-confirmed by the University of Massachusetts Medical School Nucleic Acid Facility.

Antibodies

Anti-myc polyclonal antibody has been described elsewhere (Gee et al., 1997). Anti-HA polyclonal and monoclonal and anti-myc monoclonal antibodies were purchased from Covance. Human CREST autoimmune antiserum was provided by Bill Brinkley (Baylor College of Medicine, Houston, TX). Anti-LIS1 monoclonal antibody was provided by Dr. Orly Reiner (Weizmann Institute, Rehovot, Israel) and has been characterized previously (Faulkner et al., 2000). Anti-CLIP-170 monoclonal antibody was provided by Holly Goodson (University of Notre Dame, Notre Dame, IN) and anti-tubulin monoclonal antibody was from Amersham Pharmacia Biotech (clone DM1A). Antibodies against Bub1, BubR1, and CENP-E were provided by Tim Yen (Fox Chase Cancer Center, Philadelphia, PA), and anti-Mad2 antibody was provided by Bonnie Howell and Edward Salmon (University of North Carolina, Chapel Hill, NC). Secondary HRP-conjugated donkey anti-mouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories. For immunofluorescence, Cy3-conjugated donkey anti-mouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories, and Alexa 488 anti-mouse and anti-rabbit antibodies were purchased from Molecular Probes, Inc.

Cell culture and transfection

COS-7 and HeLa cells were grown in DME with 10% FBS and penicillin/streptomycin (GIBCO BRL). For transient transfections, cells were plated either on a 10-cm petri dish or onto 18-mm coverslips to 70–80% confluency, and then were transfected with the appropriate amount of DNA mixed with lipofectAMINE (for 10-cm dish) or lipofectAMINE PLUS (coverslips) (GIBCO BRL). Transient expression was allowed for 40–48 h.

Immunoprecipitation

The coimmunoprecipitation procedure was described previously (Tynan et al., 2000b). The COS-7 cells were transfected with plasmid DNA, purified from either Qiagen Maxiprep or QIAprep Spin miniprep kits (QIAGEN), and LipofectAMINE reagents overnight at 37°C. 48 h after the addition of the DNA-liposome mixture, cells were washed twice with PBS and lysed with modified RIPA buffer (100 mM NaCl, 1 mM EGTA, 50 mM Tris, pH 8.0, 1% IGEPAL CA-630), containing a protease inhibitor mixture (2 µg/ml of aprotinin and leupeptin [Sigma-Aldrich] and 1 mM AEBSF [Roche Molecular Biochemicals]). Cells were then incubated on ice for 20 min and spun at 13,000 g for 10 min in a microfuge (Eppendorf). Protein G beads (Amersham Pharmacia Biotech) or flag M2 affinity resin (Sigma-Aldrich) were used for all immunoprecipitations. Extracts were mixed with RIPA-washed beads and incubated at 4°C overnight with gentle rotation. Beads were then washed three times with RIPA at room temperature, 10 min each, and boiled in 5× SDS-PAGE sample buffer for 5 min. The entire eluates with 4% supernatant were loaded into 5–15% SDS-PAGE gels and transferred onto Immobilon-P membranes (Millipore). Immunoblotting was performed with various antibodies against tags or specific proteins.

Yeast two-hybrid assay

The LexA-based two-hybrid assay (Gyuris et al., 1993) was used to assess interactions between LIS1 fragments and various dynein/dynactin subunits, including IC2C, dynamitin, HC N547C649, HC N748C907, and HC N1874C2124. All LIS1 fragments were cloned into the pEG202 bait vector by PCR and tested for auto-activation of the reporter. The LIS1 N bait construct activated the reporter in the absence of prey and was omitted in the two-hybrid assay. IC2C, dynamitin, and HC fragments were cloned into pJG4-5 prey vector by PCR. Interactions were scored using the β-galactosidase filter assay.

Immunofluorescence microscopy

For kinetochore staining, cells were either unextracted (Fig. 4 A) or extracted with 0.5% (Figs. 2 and 3) or 0.01% Triton X-100 (Fig. 5 B) (Sigma-Aldrich) for 1 min and fixed in 3% paraformaldehyde (EMS) in PHEM buffer (120 mM Pipes, 50 mM Hepes, 20 mM EGTA, 4 mM magnesium ac-

etate, pH 6.9) for 20 min at room temperature. Cells were then permeabilized with −20°C methanol for 4 min. All primary and secondary antibody incubations were performed at 37°C for 1 h. After all antibody incubations, cells were incubated with 0.1 µg/ml of DAPI (Sigma-Aldrich) for 10 min before mounting with Prolong Antifade solution (Molecular Probes, Inc.). Immunofluorescence images were obtained using a Leica DM IRBE microscope equipped with a Hamamatsu ORCA 100 CCD camera and processed by MetaMorph software (Universal Imaging Corp.). Confocal microscopy was performed with a Nikon Diaphot 200 microscope, using a Bio-Rad Laboratories MRC1000 system with a Kr/Ar laser. Each image was obtained from the total projection (20–24 stacks, 0.3 µm/step), and background subtraction was processed by the MetaMorph software after the image was pseudocolored and cropped by Adobe Photoshop® 5.5 (Adobe Systems, Inc.) and Corel Draw 9.0 (Corel Corp.).

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