

Light Intermediate Chain 1:
A Multifunctional Cargo
Binder for
Cytoplasmic Dynein 1

By Thomas Wadzinski

**LIGHT INTERMEDIATE CHAIN 1:
A MULTIFUNCTIONAL CARGO BINDER FOR
CYTOPLASMIC DYNEIN 1**

A Ph.D. Dissertation
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As I conclude my PhD work in Steve Doxsey's laboratory I would like to reflect for a moment on the journey that I have taken over the last six years. It is often true that we end up in places that we had not foreseen in the beginning and this was true for my thesis work. I started out looking at phenotypes associated with the over expression of pieces of pericentrin. I then moved to looking at the LIC1-pericentrin interaction in pericentrin assembly. This study of LIC1 lead me to find a mitotic delay phenotype with LIC1 depletion that didn't have to do with pericentrin. This journey had many twists and turns that sometimes appeared as dead ends. To move around or through these seeming dead ends I was provided with invaluable advice and support from numerous people along the way.

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Thomas L Wadzinski and Stephen J Doxsey, "Cytoplasmic Dynein Light Intermediate Chain 1 is Required for Progression Through the Spindle Assembly Checkpoint"

Reply from editor recommended resubmission after a few experiments are done. We are currently working on these experiments to resubmit this manuscript.

Abstract

Cells as dynamic, interactive, and self contained units of life have a need for molecular motors that can create physical forces to move cargoes within the cell. Cytoplasmic dynein 1 is one such molecular motor that has many functions in the cell. The number and variety of functions that involve cytoplasmic dynein 1 suggest that there are a number of different binding sites on dynein for different proteins. Cytoplasmic dynein 1 is a multiprotein complex made up of six different subunit families. The many different combinations of subunits that could be used to make up a cytoplasmic dynein 1 holocomplex provides the variety of different binding sites for cargoes that can be individually regulated.

The following chapters flush out how light intermediate chain 1 (LIC1), a subunit of cytoplasmic dynein 1, is involved with multiple dynein functions involving the binding of different cargoes to the cytoplasmic dynein 1 holocomplex, and how the binding of these cargoes can be regulated. First, LIC1 is found to be involved in the spindle assembly checkpoint. LIC1 appears to facilitate the removal of Mad1-Mad2, a complex important in producing a wait anaphase signal, from kinetochores. Second, the involvement of LIC1 in the spindle assembly checkpoint requires the phosphorylation of LIC1 at a putative Cdk1 phosphorylation site. This site is located in a domain of LIC1 that binds various proteins suggesting that this phosphorylation could also regulate these interactions. Third, LIC1 is involved in the centrosomal assembly of pericentrin, an important centrosomal protein. From the data presented herein, LIC1 is shaping up as a multifunctional cargo binder for cytoplasmic dynein 1 that requires regulation of its various cargoes.

Chapter 1: Introduction to Dynein Function and Regulation

This chapter introduces the major topics and proteins that will be discussed in detail in subsequent chapters. It starts with looking at dynein and all of its subunits as well as the regulation of this motor. The next two sections in this chapter introduce the centrosome and the spindle assembly checkpoint in general as chapters two and three explore the involvement of dynein light intermediate chain 1 in these two areas.

Dynein

Dynein is a multiprotein motor complex that can transport many different cargoes along the microtubule cytoskeleton in the cell as well as pull microtubules when bound to a more permanent structure. Dynein is generally classified according to its largest subunit the heavy chain. The functions of the different dyneins appear to be divergent and generally non-overlapping. There are around fourteen different vertebrate dynein heavy chain genes (Asai and Wilkes, 2004). The majority of these dynein forms are used for motility of cilia and flagella, and are denoted as axonemal dyneins. Cilia and flagella are organelles that are made up of an organized array of long protofilaments known as microtubules which are anchored at the base of this structure. Different axonemal dynein forms are used to slide one microtubule in this structure along another microtubule. The axonemal dyneins and therefore the sliding of microtubules have been implicated in the beating or twisting of flagella or cilia, however there are many factors that affect this

process and so it is still unclear how the sliding of microtubules is orchestrated to result in the very smooth wave forms associated with beating flagella (Cosson, 1996; Wargo et al., 2004; Woolley, 2000).

The other two dynein forms are denoted as cytoplasmic dyneins and are named cytoplasmic dynein 1 and 2 (Pfister et al., 2005). Cytoplasmic dynein 2 has a role in cilia formation and maintenance (Pazour et al., 1999; Pfister et al., 2006; Signor et al., 1999), and recently has been suggested to have a role in Golgi localization in mammalian cells (Grissom et al., 2002) although this function is not seen in *Chlamydomonas* (Hou et al., 2004; Pazour et al., 1999). Cytoplasmic dynein 1 appears to be the form of dynein which is required for most dynein related movements of cargo within the cytoplasm. It is interesting to contrast this one motor with the kinesin family which also is required for different cargo transport within the cell (Goldstein, 2001). The kinesin family has evolved many different kinesin genes to target the many kinesin related cargoes within the cytoplasm. Dynein on the other hand appears to have one motor domain gene that targets almost all the different dynein related cargoes within the cytoplasm. It is unclear evolutionarily why dynein and kinesin motors have evolved in these different ways (Goldstein, 2001).

On the structural level kinesins are made up of one protein that has both the motor domain and the cargo binding domains. Cytoplasmic dynein 1 is made up of two copies of each of six polypeptides: dynein heavy chain 1, intermediate chains, light intermediate chains and three classes of light chains (figure 1B) (See Pfister et al., 2006 for review of

subunits). These different subunits were first classified by their molecular weight (figure 1A). Dynein heavy chain 1 is the motor domain and the other three different subunits are thought to play roles in cargo binding. Each of the non-heavy chain subunits of cytoplasmic dynein 1 have at least two fairly homologous but different genes that can be used to make up the more than one megadalton dynein holocomplex, thus allowing for some diversity in the cargo binding sites.

Dynein heavy chain 1 (*DYNC1H1*) is the largest subunit of cytoplasmic dynein 1 at around 550 kilodaltons. This subunit contains the microtubule binding sites as well as the motor domain, a cluster of six AAA domains or ATPase associated with diverse cellular activities domains. The first four of these domains are associated with p-loop motifs or Walker A motifs which can bind ATP, making these AAA domains able to both bind and hydrolyze ATP (Takahashi et al., 2004). It also contains microtubule binding sites which allow for the heavy chains to interact with this cytoskeletal element. The dimerization of dynein heavy chains allows for a sort of walking motion for dynein to move along microtubules with the two microtubule binding domains (Burgess et al., 2003).

Intermediate chains for cytoplasmic dynein 1 are around 74 kilodaltons and there are two genes that make up this family *DYNC1I1* and *DYNC1I2*. They contain a conserved carboxy-terminal domain with WD repeats which is important for binding the heavy chain (Ma et al., 1999). The two intermediate chains are differentially localized to different anterograde compartments in the neuronal axon suggesting unique functions between these two different family members (Dillman et al., 1996), however no cargoes

have been reported to bind directly to either of the intermediate chains. The intermediate chain recruits dynein to sites of cell-cell contact through interactions with PLAC-24 and beta-catenin, both part of adherens junctions (Karki et al., 2002; Ligon et al., 2001). This anchoring of dynein allows for microtubule plus ends to be captured and placed under tension at sites of cell-cell contact, creating a dynamic interphase microtubule network centered at the centrosome where the minus ends of microtubules are anchored (Karki et al., 2002; Ligon et al., 2001). Thus, the intermediate chain has direct interactions with proteins outside of the dynein subunits but these are not really cargoes but related to sites of dynein localization.

The intermediate chain is also a platform for binding the light chains as well as dynactin. Dynactin is a multiprotein complex involved in improving dynein processivity along microtubules (Karki and Holzbaur, 1995; King and Schroer, 2000; Vaughan and Vallee, 1995). Two monoclonal antibodies to cytoplasmic dynein 1 intermediate chain 1 (74-1 and 74-2) were mapped to the amino-terminus in the region of the dynactin binding domain and appear to disrupt this interaction (Steffen et al., 1997). Both of these antibodies when used for immunodepletion experiments in *Xenopus* extracts resulted in a loss of endoplasmic reticulum network formation. Along with this loss of membrane movement, was a loss of dynein localization to membranes (Steffen et al., 1997). Further study of dynactin itself shows that disruption of its interaction with dynein results in the dispersal of Golgi and late-endosome/lysosome localization in the cell (Burkhardt et al., 1997). These studies show that dynactin binds dynein through the intermediate chain and that it is important for dynein binding to different vesicle cargoes.

The intermediate chain also connects the motor domains in the heavy chain to many different cargoes through its interactions with the light chains. The light chains appear to be small adaptor proteins, 8 to 11 kilodaltons, that allow for specific interactions. There are three different light chain gene families: Tctex, Rb and LC8. Each of these families consist of at least two known genes. The Tctex family includes two genes *DYNLT1* and *DYNLT3*. Tctex2-like sequences, or what would be *DYNLT2* genes, are only distantly related to the other Tctex family members and Tctex2 is associated with axonemal dynein and has not been definitively been shown to be part of a cytoplasmic dynein complex (Pfister et al., 2006). The Tctex family of genes has been shown to interact with a range of proteins: an ion channel, a receptor, a virus and a pigment protein (Douglas et al., 2004; Machado et al., 2003; Schwarzer et al., 2002; Tai et al., 1999). Rhodopsin, a visual pigment used in photoreceptor cells, shows the specificity between the two genes in this family as it interacts with *DYNLT1* and not *DYNLT3*. This interaction with Tctex1 is required for the dynein dependent transport of this protein (Tai et al., 1999). The specific interaction of Rhodopsin with Tctex1 shows that the subunit make up of dynein can influence its function; however no protein has been found that exclusively binds Tctex3, so the specific role of this family member remains unclear.

The Rb or roadblock family of light chain genes (*DYNLRB1*, *DYNLRB2*) have been implicated in axonal transport, mitotic function, and cell surface signaling. A mutant in a Rb family member, *robl* in *Drosophila*, results in a build-up of axonal cargo at the base of the axon (Bowman et al., 1999). This same mutant was also found to have a mitotic

defect that resulted in cells that appeared to be in anaphase or telophase with lagging chromosomes, and the great majority of the cell population appeared to be blocked in interphase as the application of nocodazole, a drug that blocks cells in mitosis, did not result in an accumulation of mitotic cells (Bowman et al., 1999). One observed interaction of human Rb1 that plays into a role in growth regulation is its association with transforming growth factor-beta (TGF β) receptors. When human Rb1 is overexpressed this signaling pathway is induced resulting in JNK activation, c-JUN phosphorylation and growth inhibition (Tang et al., 2002). The variety of functions for the Rb family from axonal transport to cell signaling and growth control suggest that Rb functions could be differentially regulated by the availability of different family members that target different cargoes. In fact, the human Rb1 and Rb2 are differentially expressed in different tissues including some cancers allowing for the possibility that dynein cargoes are altered by the differential expression of the Rb family members (Jiang et al., 2001), although the specific roles for Rb1 and Rb2 remain unclear.

The last light chain family LC8 associates with a number of different motors and enzyme complexes. There are two human members to the LC8 gene family: *DYNLL1* and *DYNLL2*. They are very homologous and initially were not distinguished from one another (Wilson et al., 2001). *DYNLL1* appears to be a more transient member of the dynein complex as large amounts are not associated with the cytoplasmic dynein 1 complex (King et al., 1996). LC8 family members, independently of cytoplasmic dynein 1, have been shown to associate with axonemal dynein, cytoplasmic dynein 2 and an actin-based motor myosin V (Espindola et al., 2000; Pazour et al., 1998; Yang et al.,

2001). It has also been shown to be involved with a few enzymes such as neuronal nitric oxide synthase used for signaling and the proapoptotic factors Bim and Bcl-2 (Day et al., 2004; Jaffrey and Snyder, 1996; Puthalakath et al., 1999). The variety of LC8 interacting protein functions certainly seems to place this gene family in a good position to have these interactions distributed among the family members to allow for more specific regulation, however these differences have not been found. DYNLL1 and DYNLL2 do not show differential binding to Bim, Bcl-2, cytoplasmic dynein 1 intermediate chain 1, or myosin V (Day et al., 2004; Naisbitt et al., 2000; Wilson et al., 2001), so it still remains to be seen how the LC8 family of genes co-ordinate their many possible interactions.

The final class of dynein subunits is the light intermediate chain and members of this family (*DYNC1L1*, *DYNC1L2*, and *DYNC2L1*) are between 50 and 65 kilodaltons. One of the light intermediate chain family members, *D2LIC* or *DYNC2L1*, associates with cytoplasmic dynein 2 and is involved in intraflagellar transport and also colocalizes with dynein 2 and the Golgi apparatus suggesting a possible role in Golgi localization (Grissom et al., 2002; Hou et al., 2004). There are two different light intermediate chain genes LIC1 (*DYNC1L1*) and LIC2 (*DYNC1L2*) that bind to cytoplasmic dynein 1. There appears to be only one LIC for cytoplasmic dynein 1 in *Caenorhabditis elegans*, *dli-1* and it has been found to be involved in mitosis as several mutant alleles of this gene resulted in failed divisions and when LIC was depleted using RNAi, problems with cleavage furrow formation were observed (Yoder and Han, 2001). LIC depletion or LIC mutants also had problems with pronuclear migration and with centrosome separation resulting in monopolar mitotic spindles (Yoder and Han, 2001). One interesting protein domain that

is conserved in the LIC family is a p-loop domain, for binding ATP, that is similar to a domain found in ABC transporters (Hughes et al., 1995). It has been suggested that this p-loop could influence ATP consumption by the heavy chain however mutation of this p-loop so that it can not bind ATP has never been shown to be required for demonstrated LIC functions (Hou et al., 2004; Tynan et al., 2000a; Yoder and Han, 2001). It may be that the p-loop is a fine tuning mechanism for dynein but loss of its function does not disable the motor.

The light intermediate chains of cytoplasmic dynein 1 create two different subgroups of cytoplasmic dynein as the dynein holocomplex has only been observed to have either LIC1 or LIC2 (Tynan et al., 2000a). There appear to be equal amounts of LIC1 and LIC2 incorporated into the cytoplasmic dynein complexes purified from calf brain white matter or COS7 monkey fibroblast cells (Hughes et al., 1995; Tynan et al., 2000a). This dichotomy suggests that dynein could be specialized for certain functions through its light intermediate chain subunit composition. In support of this idea, LIC2 and not LIC1 is localized to the fast component of retrograde axonal transport (Dillman et al., 1996), and LIC2 has been found to be up regulated when neuronal cells are stimulated to differentiate and build neurite extensions (Angelastro et al., 2000). From these two pieces of data it appears that LIC2 has a unique role in neurons separate from LIC1, however no direct interacting proteins or cargoes have been found for LIC2 that would add to this idea. LIC1 on the other hand has been found to interact with several proteins. LIC1 interacts with Rab4A, which is a GTPase involved in the regulation of membrane-receptor recycling (Bielli et al., 2001). LIC1 also interacts with pericentrin, which is a

centrosomal protein that is assembled to the centrosome by dynein (Bielli et al., 2001; Purohit et al., 1999; Tynan et al., 2000b; Young et al., 2000). Finally, LIC1 interacts with Zyg12, which is in the Hook protein family of cytoskeletal linker proteins and appears to be required for dynein localization to the nuclear envelope (Malone et al., 2003). Light intermediate chains bind dynein separately and are therefore set up to bind different cargoes to dynein, although specific LIC2 cargoes have not been identified.

From this review of the different dynein subunits it appears that there may be some differentiation of cargoes with the expression of the different dynein subunit family members. The best examples of this come with the differences seen in the fast versus slow compartments in anterograde transport compartment in axons. The different forms of the intermediate chain and the light intermediate chain are both differentially distributed between fast versus slow compartments in anterograde transport, it is thought that different cargoes can be associated with the two compartments through the different dynein subunits (Dillman et al., 1996). Another direct example of a difference between two forms of the same subunit is that light intermediate chain 1 binds pericentrin, a centrosomal scaffold protein, but light intermediate chain 2 does not (Tynan et al., 2000b). Besides these examples there are no other real comparisons of two different forms of a dynein subunit to contrast their interaction or functions. It is interesting to note that depending on the cell line there can be very different expression of the different genes of a particular dynein subunit (Pfister et al., 1996) suggesting that each subunit family member may be able to provide some basic functions but are more adept at targeting a few more specialized cargoes.

The other end of the cargo targeting question is the availability of the cargo. So even though LIC2 may bind specific cargoes for anterograde transport in the neuron, this may be due primarily to the recruitment of dynein in general to the axon and the availability of axonal cargoes that bind LIC2. This same dynein with LIC2 may be used for different cargoes in the cell body of that same neuron simply because it is in a different microenvironment that presents it with different cargoes which bind LIC2. To summarize this point some of the dynein cargo and functional differences between cell types may have to do with the availability of different cargoes and the recruitment of dynein to specific microenvironments, not with the subunit make up of dynein itself.

The targeting of dynein using different subunits may explain general differences in dynein function between different cell types but it does not explain rapid changes in dynein function that are seen in cells moving through the cell cycle or in response to cell surface signaling. These kinds of changes have usually been associated with post-translational modifications. There are several ways a phosphorylation event can change the function of dynein. The first type of phosphorylation event results in a gain or loss of a dynein subunit with implications for a gain or loss of cargo targeting. The light chain Rb1 can be phosphorylated by the transforming growth factor-beta receptor resulting in Rb1 binding to cytoplasmic dynein (Tang et al., 2002). The addition of Rb1 then changes the cargo targeting properties of the dynein population local to the receptor stimulation. A second example of regulation by phosphorylation is in a preliminary report that suggests that c-abl, a mitotic kinase, phosphorylates dynein intermediate chain 1 at Y130 (Whyte

et al., 2005). This phosphorylation of the intermediate chain is in the middle of the binding domain for the dynein light chain LC8 family and results in the loss of this subunit from the dynein complex (Whyte et al., 2005). This is suggested to allow phosphorylated dynein to localize to the cortex of cells in late mitosis and then to the congressing furrows of cells undergoing anaphase and cytokinesis. A third example is phosphorylation of intermediate chain 1 at S84 (Vaughan 2001). This phosphorylation site is localized within the binding site for dynactin and appears to disrupt the interaction of dynactin with dynein. As dynactin is involved in targeting dynein to membranous cargoes the removal of dynactin would result in the loss of this cargo capacity. These examples show the gain or loss of dynein subunits with phosphorylation.

A second type of phosphorylation event results in a gain or loss of dynein functionality but not targeting. One example of this is a general observation with an *in vitro* endoplasmic reticulum (ER) assay in which dynein moves ER tubules creating a network. When this assay was performed in the presence of phosphatase inhibitors, thus increasing or at least maintaining phosphorylations, more ER movement was observed. Total dynein bound to the ER did not change suggesting that a phosphorylation event increased the ability of dynein to move along microtubules, not the ability of dynein to target its cargo (Allan, 1995). This example demonstrates how a phosphorylation event can result in a gain or loss of dynein functionality without affecting the targeting of dynein.

The third type of phosphorylation event directly affects the binding site on a dynein subunit for a particular cargo. The one example of this that during the transition from

interphase to mitosis dynein is observed to lose its affinity for vesicle cargoes (Addinall et al., 2001). Cyclin dependent kinase 1 (cdk1 or cdc2-cyclin B1) is a major mitotic kinase and it has been shown to phosphorylate light intermediate chain 1 in more than one position in mitotic extracts (Addinall et al., 2001; Dell et al., 2000). These phosphorylations of light intermediate chain 1 have been implicated in this transition from interphase dynein binding to many membranous cargoes to mitotic dynein which binds far fewer membranous cargoes (Addinall et al., 2001). The mechanism of this change is not clear as light intermediate chain 1 has not been shown to be a pivotal subunit in binding membranous cargoes to dynein. Another possibility is that the cargo, in this case the vesicles, have been altered in the transition from interphase to mitosis and then no longer interact with dynein. While this example is not the strongest example it demonstrates the idea of how a phosphorylation event could affect the binding of cargoes but not the overall dynein functionality or subunit make-up.

To conclude, the expression of different levels of dynein subunits may set up a certain population of dynein holocomplexes. Between cells these populations of dynein may differ resulting in different cargo selections and functions. This basic cargo targeting can then be altered quickly by post-translational modifications such as phosphorylation in response to signaling or cell cycle changes.

Cytoplasmic dynein 1 light intermediate chain 1: a sequence based case study of differential targeting

The protein structures of the light intermediate chains and their cargo interacting domains give insight into the different kinds of cargo regulation for dynein described previously in

this chapter. The protein sequences of LIC1 and LIC2 are very well conserved across species (figure 2A). For example human LIC1 is 90% identical to rat LIC1, 85% identical to chicken LIC1, and 78% identical to *Xenopus* LIC1 (figure 2B). LIC1 is also very similar to LIC2 as can be observed comparing these two genes within one species. For example within humans LIC1 and LIC2 have 61% identical and 74% homologous amino acids in their protein sequence (figure 2B). If these two LIC family members are to engage different cargoes then there should be some regions in which they are not homologous where different cargoes can be targeted. Indeed, comparing 25 amino acid sections of rLIC1 to rLIC2 there are a few regions where there is comparatively low homology: rLIC1 AA1-25 and AA201-225 (figure 3A). The AA201-225 region of rLIC1 is part of the binding domains for both pericentrin and Rab4A (Bielli et al., 2001; Tynan et al., 2000b), supporting the idea that LIC1 targets cargoes through these areas of low homology with LIC2 (figure 3B). LIC2 would presumably target other cargoes through its area of low homology with LIC1, thus allowing dynein the ability to differentially associate with cargoes depending on its association with LIC1 or LIC2.

Another interesting point here is that the one well demonstrated phosphorylation site on LIC1 is right in the middle of the AA201-225 region of low homology and cargo targeting. LIC1 has been shown to be phosphorylated by cdk1 at S207 by mutational analysis and mass spectrometry (Addinall et al., 2001; Dell et al., 2000). The cdc2 site in light intermediate chains (SPQR) is not disrupted in any of the sequences we have for LIC1 or LIC2 (figure 4A). This maintenance of sequence within a region that has diverged between LIC1 and LIC2 shows a strong selection to keep this phosphorylation

site intact. It also places this point of regulation within a region that at least in LIC1 is involved in binding several cargoes, allowing this phosphorylation to be involved in regulating these interactions. Looking at the other potential cdc2 phosphorylation sites, they also fall within a region of fairly low homology from AA376 to AA425 in rLIC1, suggesting that there may be other proteins that bind in this area that are regulated with these phosphorylations.

To summarize, the regions of low homology allow for different cargoes to be targeted by LIC1 or LIC2. The conserved regulatory domain within the low homology area allows for further regulation of LIC1 specific cargoes or LIC2 specific cargoes. For example if a single kinase could phosphorylate both LIC1 and LIC2, it could simultaneously affect two different cargo groups carried by LIC1 or LIC2. The other possibility here is that LIC1 and LIC2 could have different kinases targeting this phosphorylation site, as it is possible that MAPK can phosphorylate this site. These differential phosphorylations would allow for independent regulation of LIC1 and LIC2 cargoes. Low homology areas between light intermediate chains and phosphorylation sites within these low homology regions allows for greater diversity and control of dynein cargo targeting.

Centrosome

The centrosome is a small perinuclear organelle made up of two centrioles around which there is a dense, partially filamentous matrix called the pericentriolar material or PCM (Brinkley, 1985; Doxsey, 2001). The PCM is involved in both nucleating and anchoring microtubules, allowing for the focal organization of microtubule minus ends at the centrosome in interphase cells. The plus ends of microtubules extend to the periphery of

the cell and provide a vast network of “tracks” for molecular motors to transport information, molecules and vesicles to and from the cell surface and the nucleus. As a hub for communication in the cell there are more than one hundred different regulatory or signaling proteins and kinases that can localize to the centrosome (Doxsey et al., 2005b). Some of these proteins are involved in signal transduction pathways such as MAPK and Wnt signaling. There are also proteins involved in the cellular response to different types of stress such as apoptosis and DNA damage checkpoint proteins. Still others are involved in the regulation of the cell cycle (Doxsey et al., 2005a; Doxsey et al., 2005b). A more physical manifestation of the centrosome as a hub for communication is that the PCM and one centriole form the base of the primary cilia, a sensory organ that protrudes from the surface of a cell (Hiesberger and Igarashi, 2005). From these studies, the PCM of the centrosome seems to be a hub for connecting incoming signals with outgoing cellular responses and regulation.

Aberrant PCM size, number and organization have been implicated in the occurrence of aneuploidy in cancer cells. These kinds of defects could affect how the cell responds to different environmental and cell cycle cues complicating the physical problems that multiple centrosomes can present to a dividing cell. The centrosome duplicates once in a cell cycle and is important for establishing a bipolar spindle, with a single centrosome at each pole, that aids in the faithful segregation of chromosomes and, over multiple divisions, the genome stability of a cell line (Doxsey, 2002). Along this line carcinomas and pre-invasive carcinomas have centrosomal abnormalities and genetic instability

(Pihan et al., 2001; Pihan et al., 2003) suggesting that problems with centrosome structure or assembly could have an impact on genetic stability.

Pericentrin is a large coiled-coil protein and is a component of the PCM (Doxsey et al., 1994). Pericentrin appears to act as a scaffold at the centrosome both structurally (Dictenberg et al., 1998) and molecularly to possibly increase signaling or reaction efficiencies by localizing kinases and other proteins close to one another. Structurally, pericentrin forms a very intricate lattice network at the centrosome along with other proteins (Dictenberg et al., 1998). Molecularly, pericentrin appears to have several roles. First, pericentrin is part of a microtubule nucleating complex along with gamma-tubulin which is recruited to the centrosome through the cell cycle so that greater nucleating capacity is possible in mitosis (Dictenberg et al., 1998; Kellogg et al., 1994; Kuriyama and Borisy, 1981; Young et al., 2000). Second, pericentrin is also a docking site for protein kinase C and cAMP dependent kinases, thus facilitating the signaling of these pathways at the centrosome (Chen et al., 2004; Diviani et al., 2000). Pericentrin appears to be an integral centrosome protein as it has an influence on many of the general functions of the centrosome from microtubule nucleation and organization to facilitating signaling networks at the centrosome.

The assembly of pericentrin to the centrosome therefore influences a number of different factors in the cell and if not controlled could lead to disruption or deregulation of signaling pathways and microtubule organization, especially important for mitotic spindle organization and genome stability (Chen et al., 2004; Pihan et al., 2001; Purohit et al.,

1999). Pericentrin was found to assemble onto the centrosome through the cell cycle, starting from low levels in G1 and reaching maximal levels in metaphase. This assembly is followed by a disassembly in anaphase and telophase to reach low levels again in early G1 (Dictenberg et al., 1998). Pericentrin was found to interact with the molecular motor dynein and also move at dynein like speeds (Purohit et al., 1999; Tynan et al., 2000b; Young et al., 2000). Disruption of microtubules or the dynein motor complex resulted in pericentrin being unable to assemble to the centrosome (Young et al., 2000). This evidence implicates dynein in the assembly of pericentrin to the centrosome. As pericentrin interacts with LIC1, the LIC1-pericentrin interaction is a clear candidate for how pericentrin associates with dynein for assembly but this has not been validated.

Spindle assembly checkpoint

To maintain genetic stability, cells must divide their replicated chromosomes equally between two daughter cells. Segregation of replicated chromatids occurs as cells progress from metaphase to anaphase. This transition is monitored by the spindle assembly checkpoint (Hoyt et al., 1991; Li and Murray, 1991; McIntosh, 1991; Rieder et al., 1994; Zhou et al., 2002). This checkpoint appears to monitor two parameters: microtubule attachment to kinetochores and tension across sister kinetochores (Rieder et al., 1994; Zhou et al., 2002). It is difficult to separate these parameters and to determine if they provide two independent inputs to the spindle assembly checkpoint because they are structurally dependent: attachment is needed for tension and attachment is actively strengthened with tension (King and Nicklas, 2000). What is clear is that the spindle assembly checkpoint ensures that sister chromatids are correctly oriented (i.e. attached and under tension) so that they can be segregated equally upon anaphase onset.

The initial proposal – that an unattached kinetochore produces a wait anaphase signal (McIntosh, 1991) – is supported by studies in which laser ablation of the last unaligned kinetochore allows cells to progress to anaphase (Rieder et al., 1995). This last unaligned kinetochore inhibits the onset of anaphase by preventing the activation of the anaphase promoting complex/cyclosome (APC/C)(Murray, 2004; Peters, 2002). The APC/C is an E3 ubiquitin ligase and when activated targets securin and cyclin B for proteolysis.

Securins indirectly hold the sister kinetochores together, and their degradation allows for sister kinetochores to move to opposite poles in anaphase. Cdk1 (cdc2-cyclin B) is the major mitotic cyclin and its activity inhibits the onset of anaphase. Once cyclin B is degraded, or when cdk1 is inhibited, cells will move into anaphase even when protein degradation has been blocked (Chang et al., 2003; D'Angiolella et al., 2003; Potapova et al., 2006). There are two activators of the APC/C: Cdc20 and cdh1. Cdc20 has been shown to activate APC/C-mediated degradation of securins and cyclin B to initiate anaphase (Hagting et al., 2002). The spindle assembly checkpoint inhibits the onset of anaphase by binding and sequestering Cdc20.

Early genetic screens looking for proteins involved in arresting cells treated with microtubule depolymerizing drugs revealed six different genes: Mad 1-3 and Bub 1-3 (Hoyt et al., 1991; Li and Murray, 1991). Most of these proteins have kept their yeast names when their human homologues were found. BubR1 is the homologue of Mad3 with an added kinase domain. Since the initial discovery of these genes, others proteins have been implicated in the functioning of the spindle assembly checkpoint such as

dynein, Mps1, Ndc80 complex, Rod/Zw10/Zwilch complex, and aurora kinase (Howell et al., 2001; Taylor et al., 2004). The following discussion will separate these and other spindle assembly checkpoint related proteins into several groups: the best signaling candidates, structural components, kinases and finally motor proteins.

Two proteins have been found to directly bind Cdc20 and therefore able to sequester Cdc20 from the APC/C: Mad2 and BubR1 (Fang, 2002). Both of these proteins along with Cdc20 and Bub3 are part of the mitotic checkpoint complex or MCC. This complex can inhibit the APC/C suggesting that inhibition of the APC/C is more than just sequestration of the activator Cdc20 (Sudakin et al., 2001). Both BubR1 and Mad2 are needed for an active spindle assembly checkpoint (Gorbsky et al., 1998; Meraldi et al., 2004). Mad2 has been shown to be a good indicator of microtubule attachment as it localizes to unattached kinetochores but then moves off once there is adequate microtubule attachment (Howell et al., 2000; Howell et al., 2001; Waters et al., 1998). BubR1 has been shown to be a good indicator of tension across sister kinetochores as it localizes to sister kinetochore pairs until they are placed under tension (Chan et al., 1999; Hoffman et al., 2001; Skoufias et al., 2001). Both Mad2 and BubR1 appear to be the best candidates for signaling that a kinetochore is not attached or under tension and inhibiting the APC/C as they are good indicators of attachment or tension and they interact directly with Cdc20 the activator of the APC/C.

The second group of checkpoint proteins have been shown to be important in structurally setting up the checkpoint. These proteins are required for the attachment of other

checkpoint proteins and for the localization of Mad2 or BubR1 to unattached kinetochores. Bub3 interacts with BubR1 and is required for the localization of BubR1 to kinetochores (Meraldi et al., 2004; Taylor et al., 1998). This role of Bub3 is structural but it may have a larger role as part of the MCC in inhibiting the APC/C, but this has not been looked at in detail (Sudakin et al., 2001). Several proteins have been shown to be required for Mad2 localization to kinetochores: Bub1, Rod/Zw10/Zwilch complex, Mad1 and the Ndc80 complex (Buffin et al., 2005; Chen et al., 1998; DeLuca et al., 2003; McClelland et al., 2003; Meraldi et al., 2004). Most of these proteins also have at least one additional role in the checkpoint. Mad1 appears to play a role in priming Mad2 at the kinetochore for interacting with Cdc20 (De Antoni et al., 2005). The Rod/Zw10/Zwilch complex is needed for dynein localization to the kinetochore (Karess, 2005). The Ndc80 complex appears to be a central structure because without it a number of proteins besides Mad2 do not localize to kinetochores: Rod, Zw10, dynactin, Mad1 and possibly Bub1 and Bub3 (DeLuca et al., 2003; McClelland et al., 2003; Meraldi et al., 2004). The Ndc80 complex also appears needed for good microtubule attachments to be made at the kinetochore (DeLuca et al., 2005). These proteins show how structurally complex the kinetochore is and there is also the suggestion that each of them has a distinct non-structural function to play within the checkpoint.

It is not surprising that with the abrupt and vast changes to cell morphology entering mitosis and even within mitosis that there are post-translational modifications that help to regulate these changes. Some of these modifications are phosphorylations by kinases, the third group of proteins. The most overarching kinase in mitosis is probably Cdk1. This

kinase promotes entry into mitosis and maintenance of structures in mitosis through the phosphorylation of multiple substrates which leads to nuclear membrane breakdown, assembly of the mitotic spindle, and condensation of chromosomes (Ferrari, 2006). Cdk1 also sets up its own inactivation by placing control of Cdc20 under the regulation of the spindle assembly checkpoint (D'Angiolella et al., 2003). When all sister kinetochores are attached and under tension, the spindle assembly checkpoint no longer sequesters Cdc20 resulting in the activation of the APC/C, the destruction of cyclin B and the inactivation of Cdk1. Polo-like kinase (Plk1) regulates certain aspects of microtubule dynamics, chromosome segregation and APC/C activation (Ferrari, 2006). Aurora B kinase appears to be involved in the tension pathway that causes cells to arrest when sister kinetochores are not under tension (Taylor et al., 2004), although the mechanism of Aurora B is still unclear as it affects both Mad2 and BubR1 localization to kinetochores (Ditchfield et al., 2003). These examples show some of the different mitotic functions that are regulated by kinases.

The dynamic nature of the bipolar spindle and the movement of proteins off kinetochores to spindle poles suggests the work of molecular motors, the fourth group of proteins. These proteins are like the structural proteins in that most are not directly associated with the major signaling proteins, Mad2, BubR1, or Cdc20 but they help to create an environment that allows the spindle assembly checkpoint to function. First there are several motors, Eg5, Kif2A and KifC1 that help to establish and maintain a bipolar spindle which is important for generating tension across kinetochores and also in the final goal of separating the duplicated DNA to two daughter cells (Zhu et al., 2005). Second

there are motor proteins, MCAK, Cenp-E, KifC1, Kif14, Kif18 and Kid that help to move chromosomes to the metaphase plate (Zhu et al., 2005). Some of these proteins have been studied in detail and their mechanism of action is a little clearer. Cenp-E is involved in resolving syntelically attached chromosomes, which have both of their kinetochores attached to one pole and signal the spindle assembly checkpoint presumably due to a lack of tension. Cenp-E helps to move these syntelically attached chromosomes from the one pole that they are attached to toward the metaphase plate to become bi-oriented (Kapoor et al., 2006). MCAK is a member of the Kinesin I family which has a microtubule motor domain but acts to depolymerize microtubules rather than a true microtubule motor that translocates across them (Desai et al., 1999; Hunter et al., 2003; Ovechkina and Wordeman, 2003). MCAK depletion in mitosis results in defective microtubule attachments such as syntelic and merotelic attachments (Kline-Smith et al., 2004). Merotelic attachments are where one kinetochore is attached to both poles. The sister kinetochore to a merotelically attached kinetochore can be normally attached to one pole resulting in a kinetochore pair that is attached and under tension at the metaphase plate but upon anaphase onset the one merotelic kinetochore that is attached to both poles cannot move to one pole or the other and is termed a “lagging” chromosome (Kline-Smith et al., 2004). The ability of MCAK to depolymerize microtubules has been suggested as a possible mechanism by which these unwanted merotelic or syntelic attachments are resolved (Andrews et al., 2004; Moore and Wordeman, 2004). The involvement of so many motor proteins in the mitotic spindle and in mitosis itself demonstrates the dynamic and fast moving nature of cell division.

The final group of motors are those that allow for movement of checkpoint proteins off of the kinetochore when under appropriate attachment and tension, thus disabling further signaling of the checkpoint and promoting the onset of anaphase. The movement of checkpoint proteins off of kinetochores appears to involve dynein (Howell et al., 2000; Howell et al., 2001). Most of the documentation for movement has been done with Mad2 but there is some evidence for a similar mechanism for BubR1. A prometaphase cell has unattached kinetochores that have Mad2 localized to them. As a cell moves from prometaphase to metaphase and microtubules attach to kinetochores, Mad2 localizes along the microtubule spindle and then to the spindle poles. It appears from this that Mad2 moves off of kinetochores, along microtubules toward the spindle pole, and in fact movements like these have been seen with live imaging of fluorescent Mad2 (Howell et al., 2000). This movement also suggests a minus end directed microtubule motor, as the minus ends of microtubule are anchored at the spindle poles. The major minus end directed microtubule motor is dynein, although there are a few kinesins that have now been shown to translocate in this direction as well. Inhibition of dynein with the injection of p50 or the intermediate chain antibody 70.1 results in the disappearance of fluorescent Mad2 transport and localization to spindle poles, showing the dependence of Mad2 on dynein for transportation from kinetochores to spindle poles (Howell et al., 2001). These experiments strongly support the idea that a functional dynein is important for moving Mad2 off of kinetochores to spindle poles.

There are further studies on the translocation of Mad2 as well as BubR1 using an experimental system that reduces ATP levels with azide, 2-deoxyglucose and oxryase

(AZ/DOG). The treatment of live cells with AZ/DOG does not appear to alter general spindle structure (Wadsworth and Salmon, 1988). AZ/DOG does appear to result in the premature movement of spindle assembly checkpoint proteins from kinetochores to spindle poles (Howell et al., 2001). During treatment Mad2 localizes to spindle poles but will relocalize to kinetochores after this treatment has been washed out. It appears that only kinetochore Mad2 is involved in the accumulation of Mad2 at the spindle pole as AZ/DOG treatment of metaphase cells, which have little kinetochore bound Mad2, results in little to no accumulation at the spindle poles, whereas treatment of prometaphase cells, which have kinetochore bound Mad2, results in Mad2 accumulation at spindle poles (Howell et al., 2000). Also this relocation of Mad2 requires microtubules because AZ/DOG treatment after microtubule depolymerization does not relocate Mad2 to the spindle pole and it remains at the kinetochore (Howell et al., 2000). Injection of p50 or 70.1 antibody, which disrupt dynein function, inhibits the translocation of kinetochore Mad2 to spindle poles with AZ/DOG treatment (Howell et al., 2001). Other proteins which translocate from kinetochores to spindle poles with AZ/DOG treatment are BubR1, dynein and cenp-E (Howell et al., 2001). From these experiments it appears that AZ/DOG treatment results in the microtubule dependent translocation of kinetochore Mad2 to spindle poles, an ideal experimental platform for looking at how Mad2 is removed from kinetochores. The one issue with this assay is that it is still unknown how it works. There is no clear explanation, and in fact the authors don't really comment on why a reduction in ATP would result in checkpoint proteins disregarding normal localization patterns and translocating to the spindle pole (Howell et al., 2000; Howell et

al., 2001). This flaw in the AZ/DOG experimental system casts some uncertainty on the strength of the conclusions reached from these experiments.

To conclude, the spindle assembly checkpoint ensures that sister chromatids are attached and under tension before anaphase onset to ensure their equal segregation. Anaphase onset is inhibited through the sequestration of Cdc20, the mitotic activator of the APC/C, by Mad2 and BubR1 and possibly the direct inhibition of the APC/C by the mitotic checkpoint complex. The complex structure at the kinetochore, results in many proteins being involved in setting up the interaction between Mad2 or BubR1 and Cdc20. There are also many motor proteins and kinases which control the construction and maintenance of a mitotic spindle. One motor protein, dynein, appears to be involved in removing Mad2 and BubR1 from kinetochores that are attached and under tension, resulting in the activation of the APC/C and the onset of anaphase, but the subunit of dynein involved in moving Mad2 or BubR1 has yet to be defined.

Summary

Dynein is a molecular motor which plays many different roles in the cell. Dynein is made up of many subunits and most of these subunits appear to play a role in regulating the binding of cargo. The next three chapters discuss the role of LIC1 in the spindle assembly checkpoint and pericentrin assembly, as well as how these interactions could be regulated.

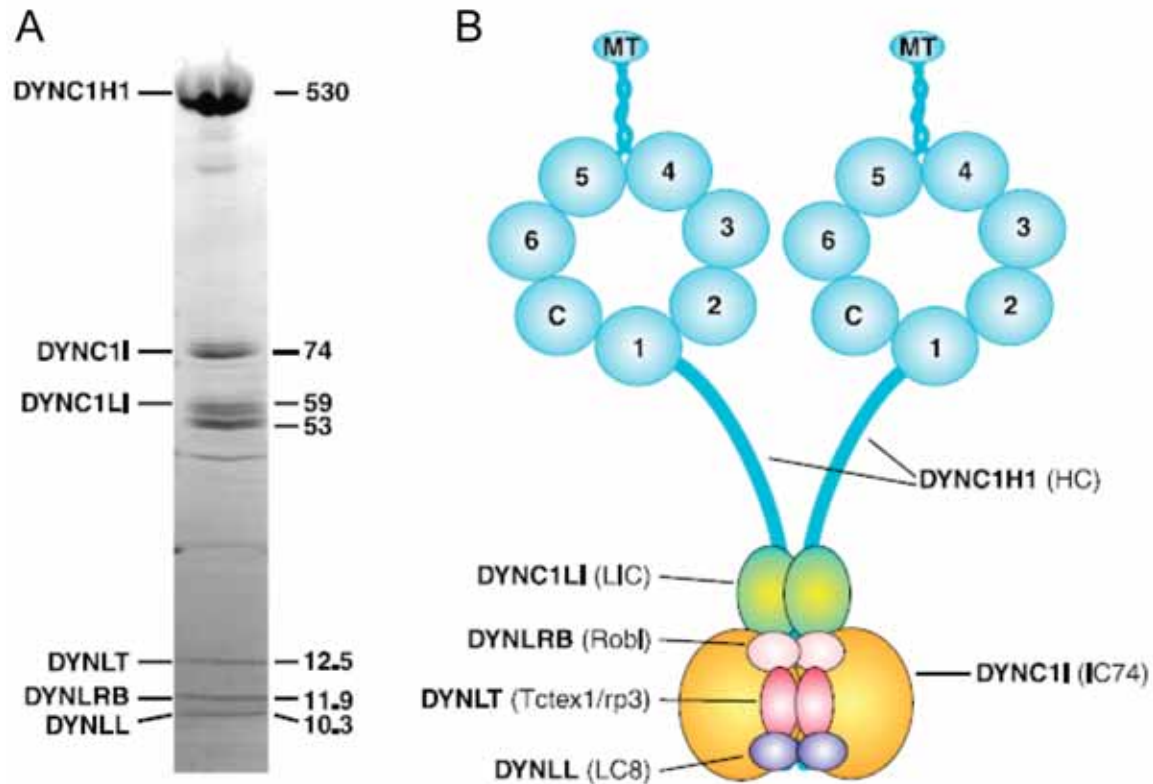
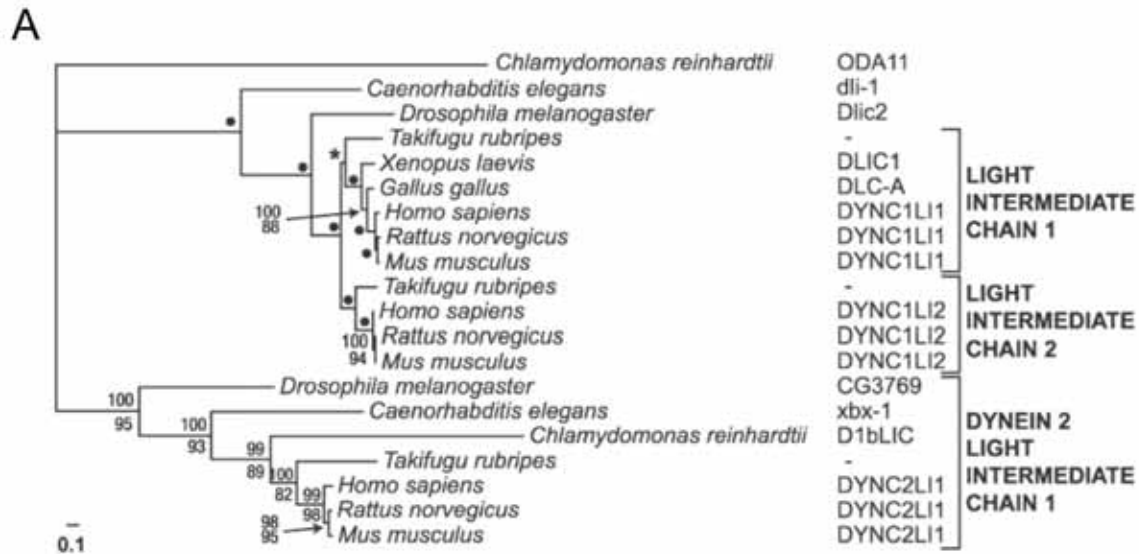


Figure 1: Mammalian cytoplasmic dynein 1 complex

A. Proteins from the cytoplasmic dynein complex purified from a rat brain prep. Approximate kilodalton mass of each peptide is listed on the right. Names of each peptide or subunit is listed on the left. DYNC1: cytoplasmic dynein 1. H1: heavy chain 1. I: intermediate chain. LI: light intermediate chain. DYN: dynein. LT: tctex light chain family. RB: roadblock light chain family. LL: LC8 light chain family. **B.** Pictorial representation of the structure of the dynein holocomplex. MT: microtubule binding site. 1-6: AAA domains. C: c-terminus of heavy chain. This whole figure is a reproduction of a previously published figure (Pfister et al., 2006).



B

Iden/tot homology	rLIC1	rLIC2	hLIC1	hLIC2	Ch DLC-A	XLIC1
rLIC1	100/100	60/73	90/93	62/75	85/91	78/89
rLIC2		100/100	59/72	93/94	62/75	62/75
hLIC1			100/100	61/74	85/91	77/87
hLIC2				100/100	64/76	62/76
Ch DLC-A					100/100	81/90
XLIC1						100/100

Figure 2: Homology between light intermediate chain sequences.

A. Protein-based phylogeny tree for dynein light intermediate chain genes. There does not appear to be a sufficiently distant homolog to these genes in *Chlamydomonas*, so ODA11 (Q39610, a heavy chain protein) was chosen as the outgroup for this tree. This graph is a reproduction of a table previously published (Pfister et al., 2006). **B.** Tabulated are the results from blast searches between the different predicted protein sequences from mRNAs in the database. The first number is the percentage of identical amino acids between the two sequences and the second number is the total number of homologous

amino acids between the two sequences. LIC: cytoplasmic dynein 1 light intermediate chain. R: rat or *Rattus norvegicus*. H: human or *Homo sapiens*. Ch: chicken or *Gallus gallus*. X: African frog or *Xenopus laevis*. DLC-A: another name for LIC1.

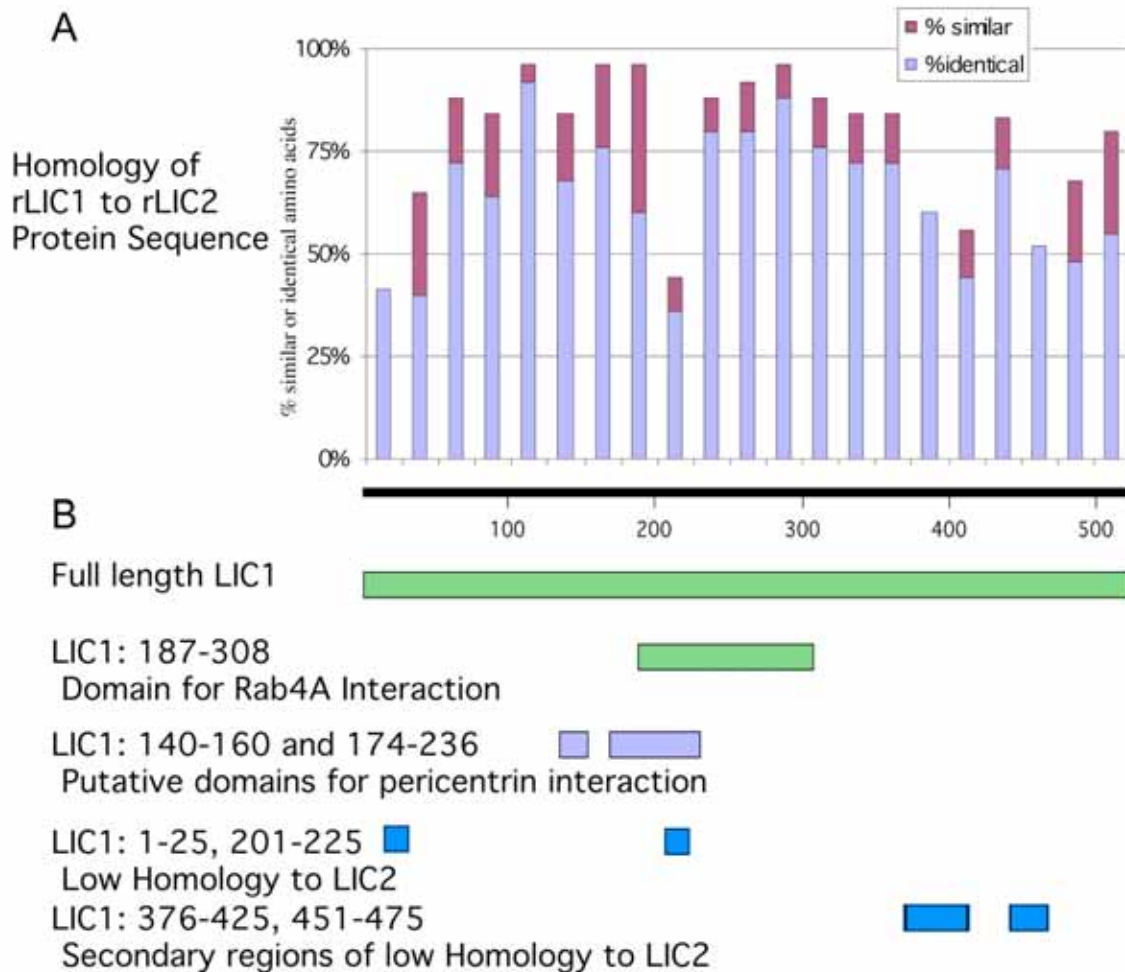


Figure 3: Map of rLIC1 and rLIC2 homology and interaction domains

A. Graph shows the percent of identical and similar amino acids when comparing 25 amino acid stretches of rLIC1 as denoted on the x-axis to the protein sequence of rLIC2.

B. Interaction domains for Rab4A and pericentrin previously described for rLIC1 (Bielli et al., 2001; Tynan et al., 2000b). Also plotted are the primary (less than or equal to 50%) and secondary (less than or equal to 60%) regions of low homology found in comparing rLIC1 with rLIC2 in part A of this figure.

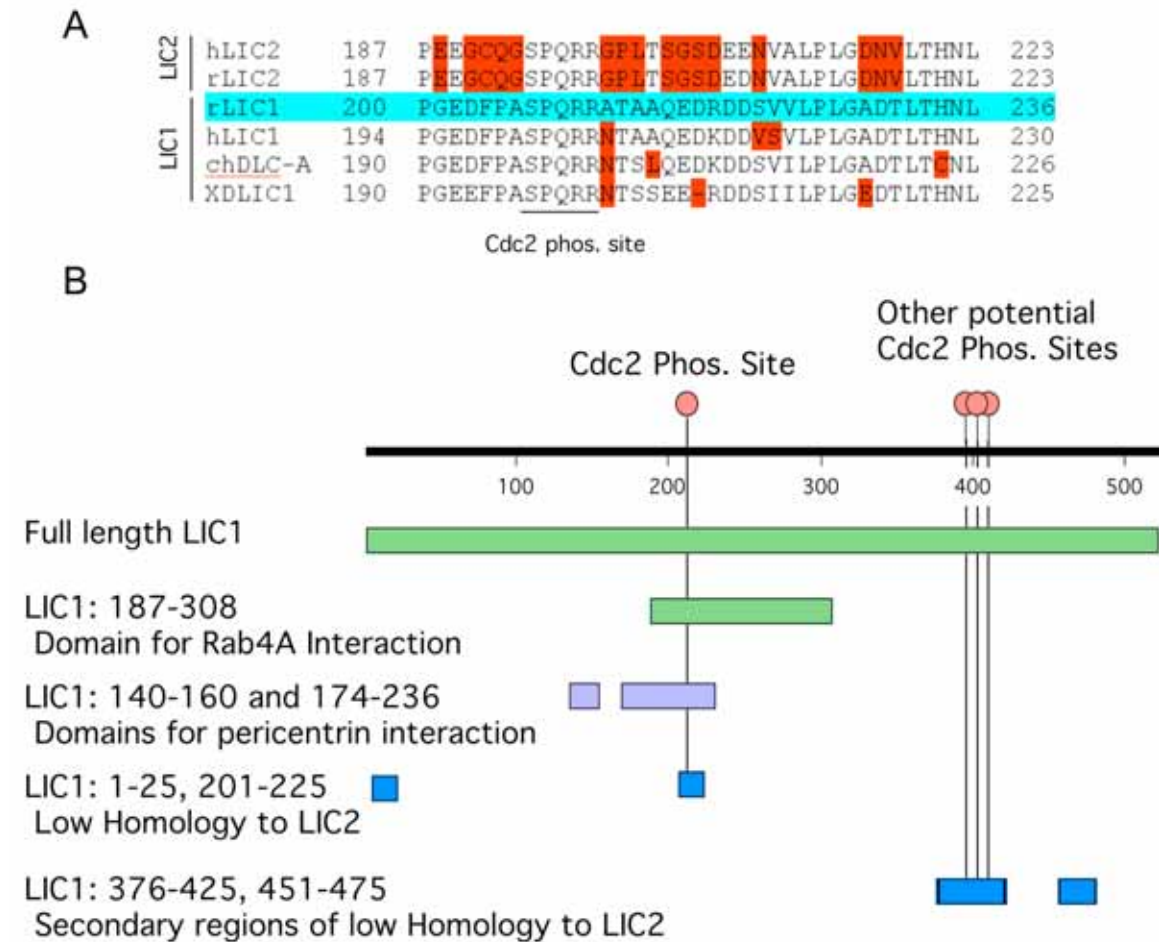


Figure 4: Sequence comparison in the pericentrin interaction domain between rLIC1 and rLIC2.

A. Alignment of LIC1 and LIC2 sequences from multiple species to amino acids 140-236 in rLIC1. Non homologous amino acids in red, compared to rLIC1 sequence in teal. R: rat or *Rattus norvegicus*. H: human or *Homo sapiens*. Ch: chicken or *Gallus gallus*. X: African frog or *Xenopus laevis*. DLC-A: another name for LIC1. Phos: Phosphorylation.

B. Map of rLIC1 phosphorylation sites and interaction domains. Cdc2 phosphorylation site at S207 was demonstrated by mass spectrometry and mutational studies and a cluster of cdc2 potential phosphorylation sites have been mapped to S398, S405 and T408

(Addinall et al., 2001; Dell et al., 2000). Interaction domains for Rab4A and pericentrin previously described for rLIC1 (Bielli et al., 2001; Tynan et al., 2000b). Also plotted are the primary (less than or equal to 50%) and secondary (less than or equal to 60%) regions of low homology found in comparing rLIC1 with rLIC2 in figure 3A.

Chapter 2: Light intermediate chain 1 of cytoplasmic dynein is necessary for progression through the spindle assembly checkpoint

Introduction

To maintain genetic stability, cells must divide their replicated chromosomes equally between two daughter cells. Segregation of replicated chromatids occurs as cells progress from metaphase to anaphase. This transition is monitored by the spindle assembly checkpoint (Hoyt et al., 1991; Li and Murray, 1991; McIntosh, 1991; Rieder et al., 1994; Zhou et al., 2002). This checkpoint appears to monitor two parameters: microtubule attachment to kinetochores and tension across sister kinetochores (Rieder et al., 1994; Zhou et al., 2002). It is difficult to separate these parameters and to determine if they provide two independent inputs to the spindle assembly checkpoint because they are structurally dependent: attachment is needed for tension and attachment is actively

strengthened with tension (King and Nicklas, 2000). What is clear is that the spindle assembly checkpoint ensures that sister chromatids are correctly oriented (i.e. attached and under tension) so that they can be segregated equally upon anaphase onset.

The precise structural and molecular underpinnings of the spindle assembly checkpoint are still unresolved. The initial proposal – that an unattached kinetochore produces a wait anaphase signal (McIntosh, 1991) – is supported by studies in which laser ablation of the last unaligned kinetochore allows cells to progress to anaphase (Rieder et al., 1995). The main molecular components required for the checkpoint, the Mad and Bub families of proteins, were originally shown to be required for the wait anaphase signal in yeast and later in humans (Hoyt et al., 1991; Li and Murray, 1991; Meraldi et al., 2004). Two members of these families, Mad2 and BubR1, function directly in the spindle assembly checkpoint by preventing degradation of cyclin B and securins and the onset of anaphase (Hoyt, 2001; Shah and Cleveland, 2000). Both localize to kinetochores and serve as indicators of an active wait anaphase signal (Howell et al., 2000; Howell et al., 2001; Waters et al., 1998) and both move off kinetochores before anaphase progression (Howell et al., 2004). Mad2 moves off kinetochores along microtubules when adequate microtubule attachments are established. BubR1 moves off kinetochores when tension across sister kinetochores is established (Chan et al., 1999; Hoffman et al., 2001; Skoufias et al., 2001). Thus, when both checkpoint criteria are fulfilled (kinetochore-microtubule attachment and inter-kinetochore tension), Mad2 and BubR1 are removed from kinetochores.

There are two pools of Mad2: one that is bound to Mad1 and one that is not (Chen et al., 1999; Shah et al., 2004; Sironi et al., 2001). Mad1 appears to be the limiting factor in the creation of the Mad1-Mad2 complex as Mad1 is always associated with Mad2 in yeast, *Xenopus* extracts and HeLa cells (Chen et al., 1999; Shah et al., 2004; Sironi et al., 2001). The only exception that has been observed is Mad1 localizes to spindle poles for longer than Mad2 does in late anaphase (Shah et al., 2004). The Mad1-Mad2 complex localizes to kinetochores through an interaction in Mad1, because Mad2 is unable to localize to kinetochores without Mad1. Thus the Mad2 that localizes to kinetochores is in the Mad1-Mad2 complex.

Further evidence supporting a Mad1-Mad2 complex at the kinetochore can be found in immunolocalization studies, FRAP studies, and biochemical studies. First, Mad1 and Mad2 both associate with kinetochores until there is microtubule attachment and then move off to spindle poles (Howell et al., 2004; Shah et al., 2004; Waters et al., 1998). This is consistent with the Mad1-Mad2 complex localizing and moving off the kinetochore as a unit. Second, the Mad1-Mad2 complex is the unit that catalyzes free Mad2 and Cdc20 into a Mad2-Cdc20 complex, a wait anaphase signal (De Antoni et al., 2005). Third, FRAP has demonstrated an immobile pool of Mad2 at mitotic kinetochores coexist with a high turnover mobile fraction (Shah et al., 2004). Mad1 is very stable with a low turn over rate presumably part of the Mad1-Mad2 complex and associated with the stable fraction of Mad2 (Howell et al., 2004; Shah et al., 2004). The high turn over fraction of Mad2 would be the free Mad2 that is catalyzed by the Mad1-Mad2 complex to bind Cdc20. To conclude these studies support that all kinetochore Mad1 is bound to

Mad2, and advance the idea that the Mad1-Mad2 complex catalyzes the interaction of Mad2 and Cdc20, sequestering Cdc20 from the APC/C and preventing anaphase onset.

While both the Mad1-Mad2 complex and BubR1 are recruited to kinetochores during checkpoint activation, their individual roles in the checkpoint are unclear. To date, there is no condition that activates the checkpoint in which one protein is absent from all kinetochores while the other is present on at least one; both are present on at least some kinetochores in cells with an active spindle assembly checkpoint. For example, both proteins localize to unattached kinetochores when microtubules are depolymerized, and BubR1 localizes to all kinetochores and the Mad1-Mad2 complex to a few when microtubules are stabilized by taxol (i.e. attachment but no tension) (Waters et al., 1998). Recently depletion of Nuf2 or Hec1 (both members of the Ndc80 complex) were shown to result in the activation of the spindle assembly checkpoint with BubR1 and low levels of the Mad1-Mad2 complex at kinetochores, but this does not rule out kinetochore associated Mad1-Mad2 complex at kinetochores as being necessary for the checkpoint (DeLuca et al., 2003). Additional experiments will be required to determine the separate roles of the Mad1-Mad2 complex and BubR1 in the spindle assembly checkpoint.

Cdk1 is the primary cyclin-dependent kinase complex in mitosis. It controls mitotic timing and ensures that anaphase onset does not occur prematurely, before sister chromatid segregation (Chang et al., 2003; D'Angiolella et al., 2003; Gorbsky et al., 1998; Murray et al., 1989). Anaphase onset is triggered only when Cdk1 activity is down regulated via cyclin B1 destruction (Chang et al., 2003; Clute and Pines, 1999; Minshull

et al., 1989; Reed, 2003). Cdk1 prevents exit from metaphase or the onset of anaphase by promoting the binding of Cdc20, the APC/C activator, to Mad2 and BubR1 (D'Angiolella et al., 2003). This provides free Mad2 or BubR1 with the capacity to inhibit the Cdc20-dependent activation of proteasome degradation and anaphase onset.

The multiprotein cytoplasmic dynein complex is a minus end directed microtubule motor involved in the spindle assembly checkpoint as well as in other mitotic functions such as spindle pole focusing and interphase functions such as Golgi complex positioning and vesicle trafficking (Corthesy-Theulaz et al., 1992; Merdes et al., 2000; Pfister, 2005). Dynein is comprised of six subunit families in animal cells including heavy, intermediate, light intermediate and three different light chain families (Pfister et al., 2006). It is required to move Mad1-Mad2 and BubR1 off kinetochores in metaphase, thus releasing the spindle assembly checkpoint and permitting chromosome segregation (Howell et al., 2001). The Rab6A' GTPase facilitates dynein-mediated movement of Mad1-Mad2 and BubR1 off kinetochores through its interaction with the p150^{Glued} subunit of the dynein-modulating dynactin complex (Miserey-Lenkei et al., 2006). Dynein light intermediate chains (LICs) are required for mitosis in *Caenorhabditis elegans* (Yoder and Han, 2001) and become phosphorylated during animal cell division by Cdk1-cyclin B1 (Addinall et al., 2001; Dell et al., 2000). This phosphorylation has been correlated with loss of the motor's interaction with vesicles (Addinall et al., 2001), although neither the mitotic role of these phosphorylation events nor the precise function of dynein LICs in mitosis have been addressed.

In this chapter, we identify human LIC1 as a novel contributor to the spindle assembly checkpoint. LIC1 depletion induces a Mad2-, Mad1-, and BubR1 dependent metaphase arrest that is suppressed by re-expressing a Cdk1 site phosphomimetic form of LIC1, but not a non-phosphorylatable form.

Results

siRNA-mediated depletion of LIC1 induces a delay in metaphase

Human LIC1 (hLIC1) function was investigated by treating HeLa cells with small interfering RNAs (siRNAs) targeting hLIC1 or GFP (control). Protein levels were detected by immunoblotting using an antibody raised to a peptide sequence in hLIC1 that reacted with LIC1 but not LIC2 (Figure 1). By 24-72 hours after transfection, hLIC1 siRNAs reduced LIC1 protein levels in HeLa cells by up to 90% of control levels (Figure 2A).

Cells depleted of hLIC1 showed an increase in mitotic cells (Figure 2B). Time lapse imaging of hLIC1 depleted cells using phase contrast microscopy revealed a prolonged delay in mitosis (compare Figure 2C with D and E). Quantitative analysis showed that hLIC1 depleted cells were delayed between nuclear envelope breakdown (NEB) and anaphase onset (Figure 2F, G). Control siRNA-treated cells spent up to 80 minutes from NEB to anaphase onset (average 34 minutes at 72h, n=35), whereas ~50% of hLIC1 depleted cells spent longer than 80 minutes (average 184 minutes at 72h, n=18) (Figure 2G).

Analysis of chromosomes by phase contrast microscopy showed that cells were delayed in metaphase with normally aligned chromosomes (Figure 2D, E, 3A). Metaphase arrest

was confirmed by immunofluorescence microscopy. Cells imaged for more than 100 minutes in mitosis then stained with a vital DNA dye (Syto 13) showed normal metaphase chromosome alignment (Figure 3B, last panel, n=10 cells, supplementary video 4). Moreover, all metaphase cells treated with LIC1 siRNA, including those delayed in mitosis, stained brightly with antibodies to mitotic cyclin B, consistent with their inability to degrade this cyclin and enter anaphase (Figure 3C, D). After prolonged metaphase arrest, some cells entered anaphase (Figure 2D, 2:20) while others lost the metaphase configuration but remained round (Figure 2E, 4:30-12:10), suggesting that they remained in mitosis with misaligned chromosomes.

The metaphase delay and increased mitotic index observed in LIC1 depleted cells was reversed by expression of the rat homologue of hLIC1 (rLIC1), which was not targeted by the hLIC1 siRNA (Figure 4A, B, C). This result demonstrated that the mitotic phenotype was specific for LIC1 depletion and ruled out off-target effects of the LIC1 siRNA. Taken together, these results demonstrate that LIC1 depletion induces a metaphase delay in HeLa cells.

Cytoplasmic dynein levels, complex formation, localization and function are unaltered in LIC1 depleted cells.

To test whether the mitotic delay induced by LIC1 depletion resulted from perturbation of cytoplasmic dynein, we examined several features of the motor. We found that levels of a core dynein component, dynein intermediate chain, were unaltered in LIC1 depleted

cells, suggesting that the dynein complex was not destabilized or degraded by loss of LIC1 (Figure 5A). The dynein complex sedimented in sucrose gradients to the same position as controls (Figure 5B), demonstrating that the integrity of the complex was not significantly altered. Dynein localized normally to unattached kinetochores in cells treated with nocodazole (Figure 5C, 50/50 control and 52/52 LIC1 siRNA treated cells) and to spindle poles in metaphase cells (Figure 5D, 50/50 control and 50/50 LIC1 siRNA treated cells). Neither Golgi complex organization (Figure 5E, F) (Corthesy-Theulaz et al., 1992) nor focusing of the spindle poles was altered in LIC1 depleted cells (Figure 5G, H) (Merdes et al., 2000). Taken together, these data demonstrate that the mitotic delay observed in LIC1 depleted cells was not due to cytoplasmic dynein disruption, mislocalization or functional abrogation. They also show that LIC1 is not required for localization of dynein to kinetochores or other cellular sites.

The mitotic arrest induced by LIC1 depletion requires Mad2, Mad1 and BubR1

To determine whether the mitotic delay induced by LIC1 depletion activated the spindle assembly checkpoint, we examined the role of the spindle assembly checkpoint proteins Mad2, Mad1 and BubR1 (Chen et al., 1998; Chen et al., 1996; Li and Murray, 1991; Li and Benezra, 1996; Meraldi et al., 2004; Waters et al., 1998). The mitotic index observed in LIC1 depleted cells was suppressed in cells co-depleted of LIC1 and Mad2 or Mad1 or BubR1 (Figure 6). As all of these proteins are required for cells to arrest when microtubules are depolymerized and kinetochores are unattached and not under tension

(Meraldi et al., 2004), the necessity of these proteins for cells to delay when LIC1 is depleted shows that the spindle assembly checkpoint is involved in delaying these cells.

Structural organization of checkpoint proteins at the kinetochore is normal in nocodazole treated LIC1 depleted cells

When kinetochore-microtubule attachments are eliminated by microtubule depolymerization, the following players of the spindle assembly checkpoint, Mad1-Mad2 and BubR1, localize to the unattached kinetochores (Chen et al., 1996; Li and Benezra, 1996; Taylor et al., 1998; Waters et al., 1998). In LIC1 depleted cells lacking microtubules Mad1 recruitment to kinetochores was not detectably different from control cells, showing that loss of LIC1 did not affect Mad1-Mad2 complex binding to unattached kinetochores (Figure 7A, 50/50 control and 53/53 LIC1 depleted cells had Mad1 at kinetochores). Similar results were obtained with BubR1 (Figure 7B, 51/51 control and 50/50 LIC1 depleted cells had BubR1 at kinetochores) and dynein (Figure 5C). These data demonstrate that the molecular underpinnings of the spindle assembly checkpoint, Mad1-Mad2 and BubR1, are intact.

Mad1 is retained on at least one kinetochore in LIC1 depleted metaphase cells

In control metaphase cells, in most cases kinetochores positive for Mad1 are also positive for LIC1, whereas Mad1 negative kinetochores are negative for LIC1. This indicates that in general LIC1 and associated dynein are on kinetochores when Mad1 is there (Figure 7C, arrows). In LIC1 depleted metaphase cells, Mad1 was present on at least one kinetochore in $78\pm 3\%$ of cells, similar to control siRNA treated cells ($71\pm 1\%$ of cells, Figure 7C, D). Because $\sim 50\%$ of these LIC1 depleted cells were delayed in metaphase (sometimes for many hours), this result demonstrates that Mad1, or the Mad1-Mad2 complex, is retained on at least one kinetochore in delayed cells. Cells without kinetochore associated Mad1-Mad2 likely enter anaphase as in Figure 2D.

BubR1 is reduced at kinetochores in LIC1 depleted metaphase cells

In contrast to the retention of Mad1 at kinetochores in LIC1 depleted metaphase cells, BubR1, which is sensitive to tension across sister kinetochores, appeared to be effectively removed from kinetochores. It was found at high levels on only a few kinetochores in $33\pm 11\%$ of LIC1 depleted metaphase cells compared to $83\pm 7\%$ in control metaphase cells (Figure 7E, F). Normally, BubR1 is not completely removed from kinetochores even when kinetochores are under full tension. Consistent with this, we see control amounts of BubR1 on kinetochores in LIC1 depleted cells; we have called these

“negative” for BubR1 in this assay (Figure 7E, middle and bottom row, and 7F) (Hoffman et al., 2001). Tension generation itself does not appear to be altered by LIC1 depletion as average interkinetochore distances in metaphase cells are unchanged (Figure 8A, B). Thus, the reduction of BubR1 from kinetochores most likely results from the prolonged metaphase delay and normal removal of this protein from chromosomes under tension. Collectively, these data suggest that LIC1 is involved in the selective removal of Mad1-Mad2, but not BubR1, from kinetochores.

Mad1 is retained on kinetochores that are under tension in delayed LIC1 depleted cells

Most LIC1 depleted cells delayed for more than 100 minutes had Mad1 localized to one or more kinetochores (Figure 8C). Closer inspection of these cells unexpectedly revealed Mad1-positive kinetochores that were under tension (Figure 8C, arrows, D). As expected, control cell kinetochore pairs positive for Mad1 were under little or no tension in prophase and metaphase (control prophase and metaphase, Figure 8D). This is in agreement with Mad1 moving off kinetochores as they are attached by microtubules and put under tension. In contrast, Mad1 positive kinetochores in LIC1-depleted metaphase-arrested cells (>100 min) were widely separated and thus under significant tension (Figure 8C, arrows, D). This shows that even after tension is established across sister kinetochores Mad1 still has not been removed from kinetochores. Together these results suggest that LIC1 is required for efficient removal of Mad1 or the Mad1-Mad2 complex from kinetochores.

Phosphorylation of LIC1 on the S207 Cdk1 site is required for spindle assembly checkpoint function

Cdks play a role in the spindle assembly checkpoint although few targets of Cdk1 phosphorylation have been identified in this context (D'Angiolella et al., 2003). Here we show that LIC1 undergoes an upward mobility shift on SDS gels in mitotic versus interphase cells (Figure 9A, left), suggesting a mitosis specific phosphorylation as previously shown for non-human forms of LIC1 (Addinall et al., 2001; Dell et al., 2000; Hughes et al., 1995). The mitotic phosphorylated form of LIC1 co-immunoprecipitated with the dynein complex, demonstrating an association with dynein in mitosis (Figure 9A, right). The shifted band was reduced to the interphase position following incubation with lambda protein phosphatase, showing that it was phosphorylation-dependent (Figure 9B). Previous studies showed that LIC1 was phosphorylated *in vitro* by Cdk1-cyclin B1 in mitosis on distinct residues and that serine S197 in chicken and *Xenopus* (corresponding to S207 in human and rat LIC1) was required for releasing dynein from interphase cell vesicles during entry into mitosis (Addinall et al., 2001; Dell et al., 2000). S207 mutated to alanine (S207A) did not undergo the full mitotic shift seen with the wild type protein, confirming S207 as a phosphorylation site in this system (Figure 9C). In contrast, the pseudophosphorylated form of rLIC1 (S207E) shifted to the wild type position (Figure 9C).

To determine if this phosphorylation site had a mitotic function, we tested its role in the spindle assembly checkpoint. We asked whether mutations in the rat LIC1 S207 site affected its ability to restore spindle assembly checkpoint activity when expressed in LIC1 depleted cells. The mitotic delay induced by LIC1 depletion was suppressed by re-expression of S207E and the wild type rLIC1 protein, but not by S207A (Figure 9D). In contrast, all three forms of LIC1 (wild type, S207E and S207A) co-immunoprecipitated with dynein (Figure 9C), demonstrating that LIC1 S207 phosphorylation did not regulate the LIC1-dynein interaction. Taken together, these results show that phosphorylation of rLIC1 at S207 is required for progression through the spindle assembly checkpoint and suggest that Cdk1 regulates this event perhaps by facilitating LIC1 binding to cargoes such as spindle assembly checkpoint proteins.

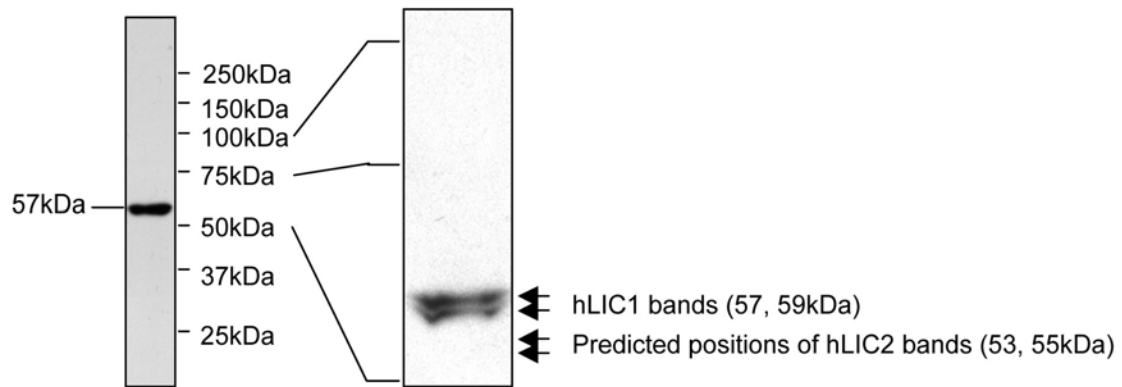


Figure 1. Anti LIC1 antibody

Immunoblot of cell lysate probed with affinity purified anti-LIC1 antibody showing a band at ~57kDa (left), which can be resolved as a doublet when gel is run longer (right, 57/59kDa) as previously seen (Hughes et al., 1995). Expected positions of LIC2 bands based on previous studies (Hughes et al., 1995) are indicated (53/55kDa).

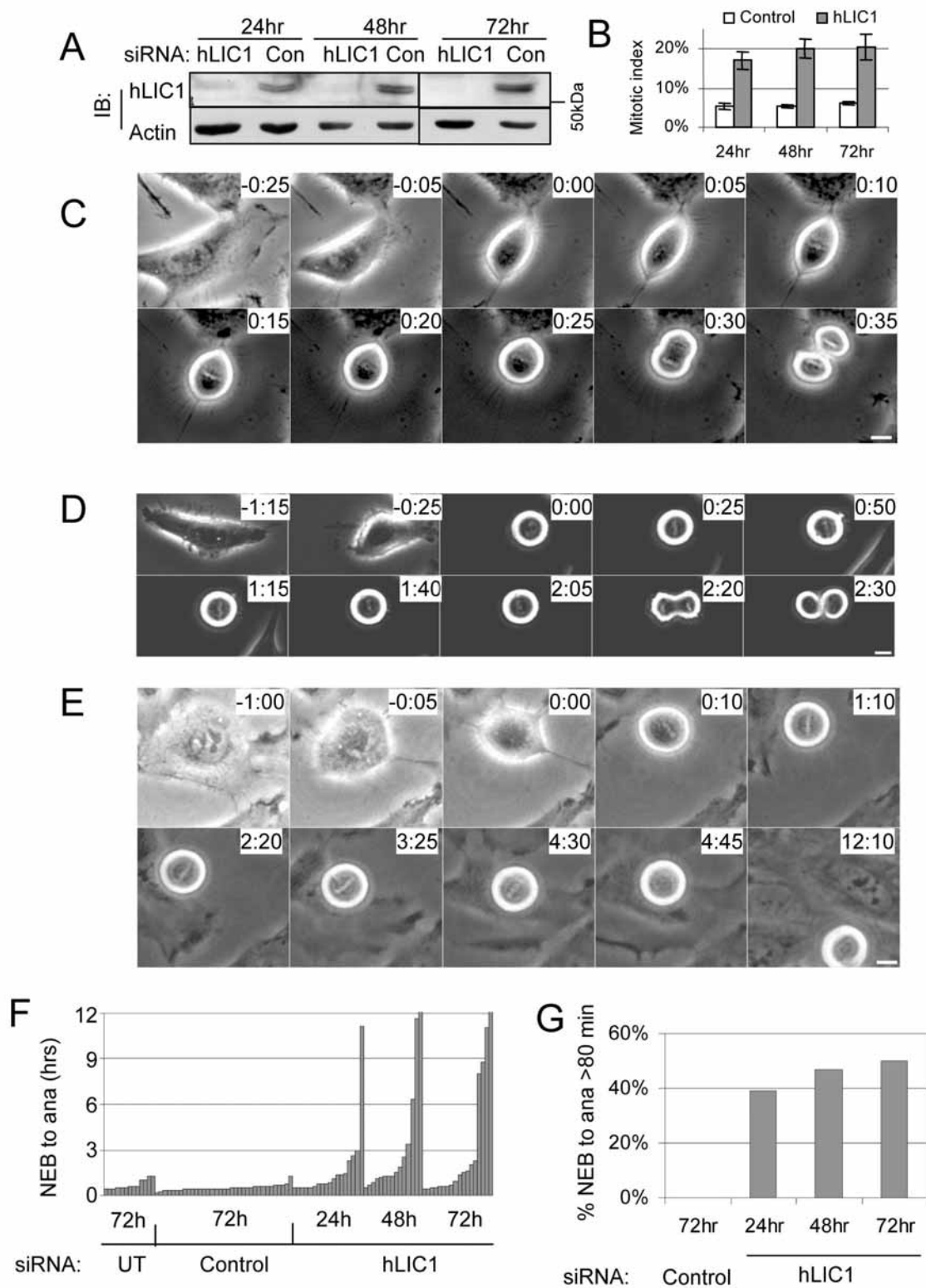


Figure 2. LIC1 depleted cells delay in mitosis

A. Immunoblot showing hLIC1 levels after treatment with control (con) or hLIC1 siRNA for the indicated times. Actin, loading control; IB, immunoblot. **B.** Mitotic index \pm SD of cells treated with control or hLIC1 siRNA for the indicated times (each bar represents 1200 cells from two experiments). **C-E.** Phase contrast images taken from a time lapse series showing a typical HeLa cell treated with control siRNA (GFP), this cell takes 30 minutes to go from nuclear envelope breakdown (NEB) to anaphase (C, 0:30) compared with 2 hours and 20 minutes for a cell treated with hLIC1 siRNA (D, 2:20) or compared with a hLIC1 siRNA treated cell that did not initiate anaphase after 12 hours but lost its metaphase plate (E, 4:45-12:10). NEB, 0:00 (hr:min), bars, 10 μ m. **F.** Timing of individual cells (each bar) from NEB to anaphase onset (ana) under the indicated conditions. Untreated, UT; control siRNA, GFP. **G.** The percentage of mitotic cells that took longer than 80 minutes from NEB to ana (n=35 cells/bar for control and n=13-18 cells/bar for hLIC1) at the indicated times.

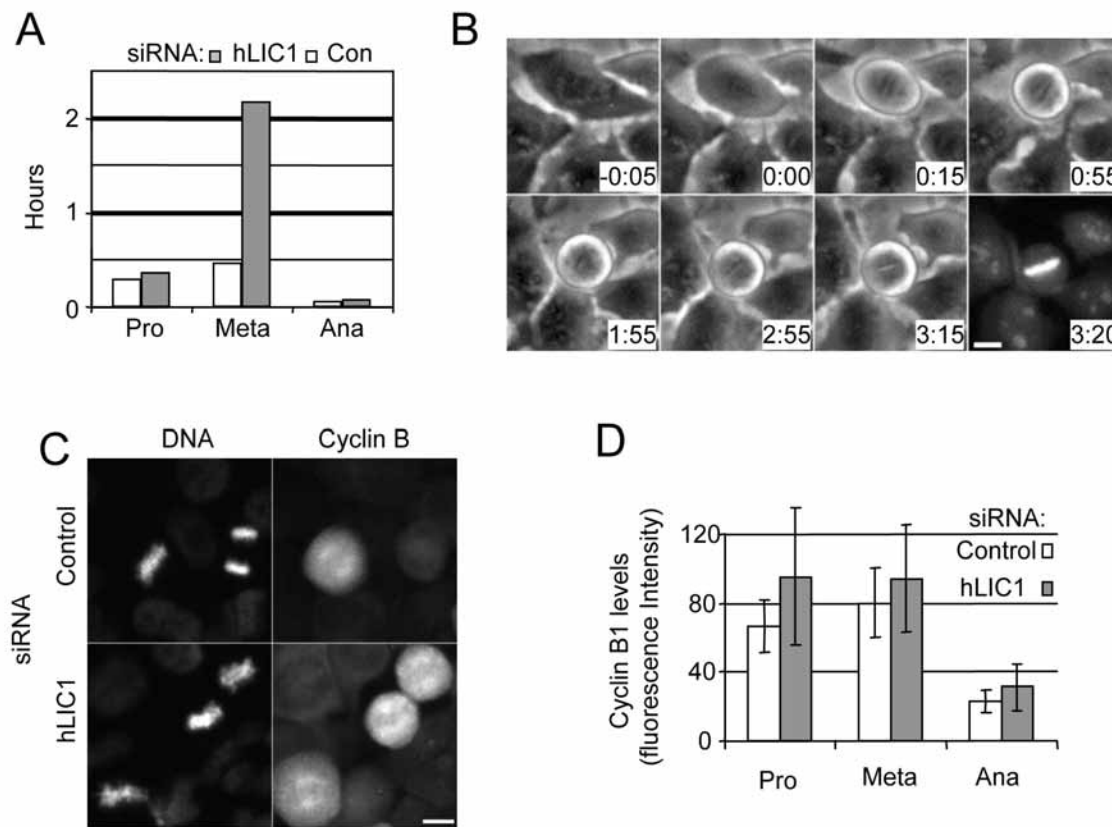


Figure 3. LIC1 depleted cells delay in metaphase

A. Average times in prophase (pro), metaphase (meta), and anaphase (ana) determined by time lapse imaging of HeLa cells treated with control (GFP, n=5 cells/bar) or hLIC1 siRNA (n=11 cells /bar). **B.** A LIC1 depleted HeLa cell forms a metaphase plate ~15 minutes after NEB (0:00) then delays in metaphase for ~3 hours. Staining with the viable DNA dye Syto13 (3:20) indicates that no chromosomes were misaligned. Bar, 10 μ m. **C.** Cyclin B immunofluorescence staining in cells treated with indicated siRNAs. DNA, DAPI stain. **D.** Average cyclin B level/cell \pm SD measured by immunofluorescence (as in

C) at the indicated cell cycle stages after indicated siRNA treatments (controls: n=9 pro, 38 meta, 12 ana; hLIC1: n=30 pro, 50 meta, 18 ana).

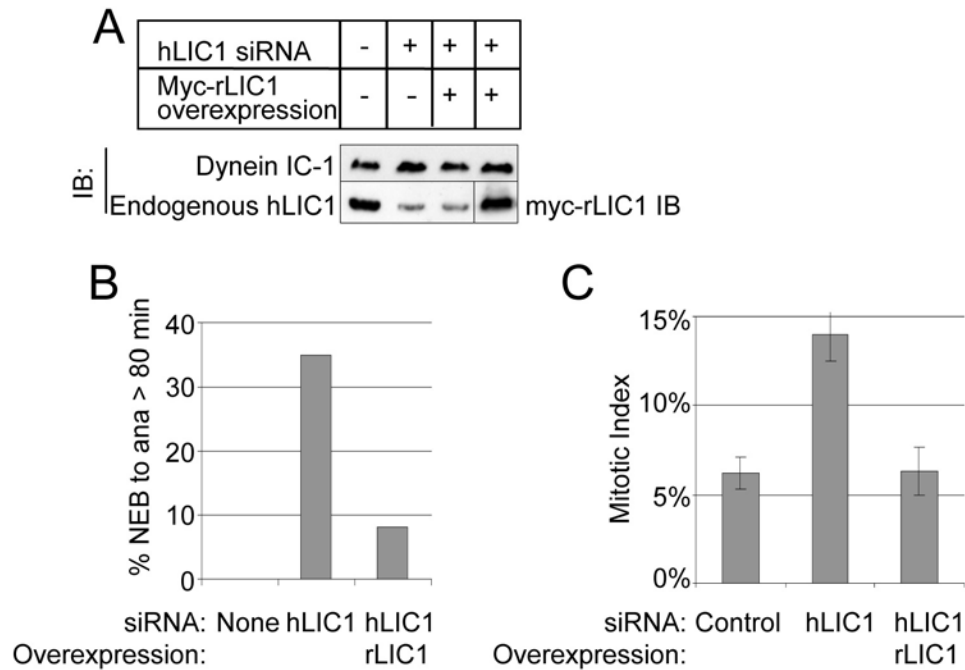


Figure 4. hLIC1 associated mitotic delay is rescued by rLIC1 overexpression

A. Immunoblot showing myc-rLIC1 overexpression in hLIC1 depleted cells. IC-1, intermediate chain 1. **B.** The percentage of cells that exceeded 80 minutes from NEB to anaphase under the indicated conditions (n= 13-17 cells/condition). **C.** Mitotic index \pm SD of cells treated with control or hLIC1 siRNA alone or a combined treatment of hLIC1 siRNA and the myc-rLIC1 construct (each bar represents 1200 cells from two experiments).

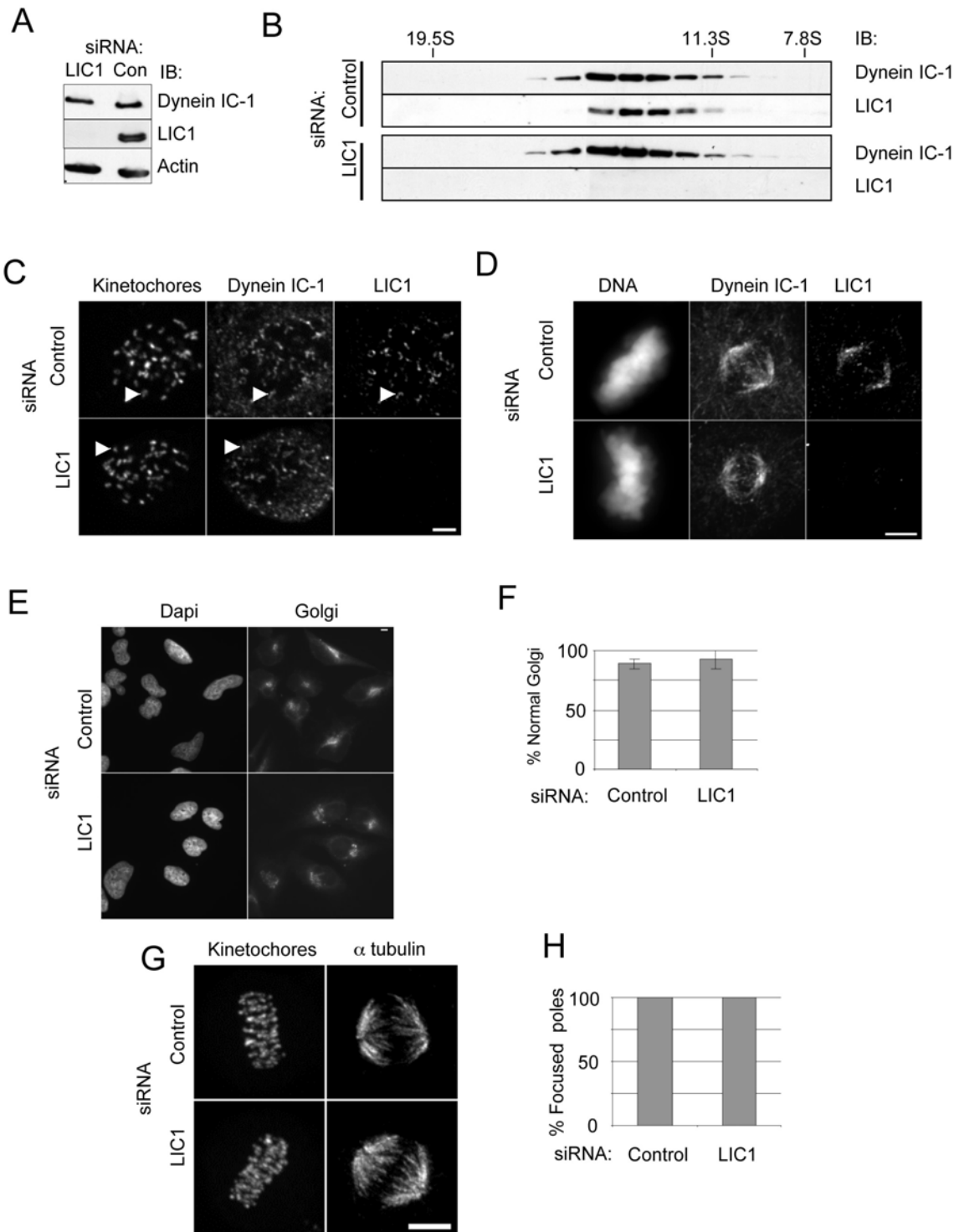


Figure 5. hLIC1 depletion does not alter dynein localization, integrity or function

A. Immunoblot of lysates from cells depleted of hLIC1 showing no significant change in dynein IC levels. **B.** hLIC1 co-sediments with dynein IC in sucrose gradients in control depletion cells. Dynein IC does not sediment differently with the depletion of LIC1. IB, immunoblot. S-value markers, above. **C.** Dynein IC localizes to kinetochores (CREST staining) in nocodazole treated control and hLIC1 depleted cells. Pictures are maximum projections of three planes, 0.3 microns apart. Arrow heads point to a typical kinetochore. Bars, 5 μ m in C, D, E. **G.** **D.** Dynein IC localizes to the spindle in control and hLIC1 depleted cells. DNA, DAPI stain. **E, F.** Golgi complex images (E) and percent focused Golgi (F) in control and LIC1 depleted cells. Bars in F, average \pm SD (control: n=129 cells from 3 experiments, LIC1: n=292 cells from 3 experiments). **G, H.** Images of control and LIC1 siRNA treated metaphase cells with focused spindle poles (G) and quantification (H, control: n=29 cells, LIC1: n=72 cells).

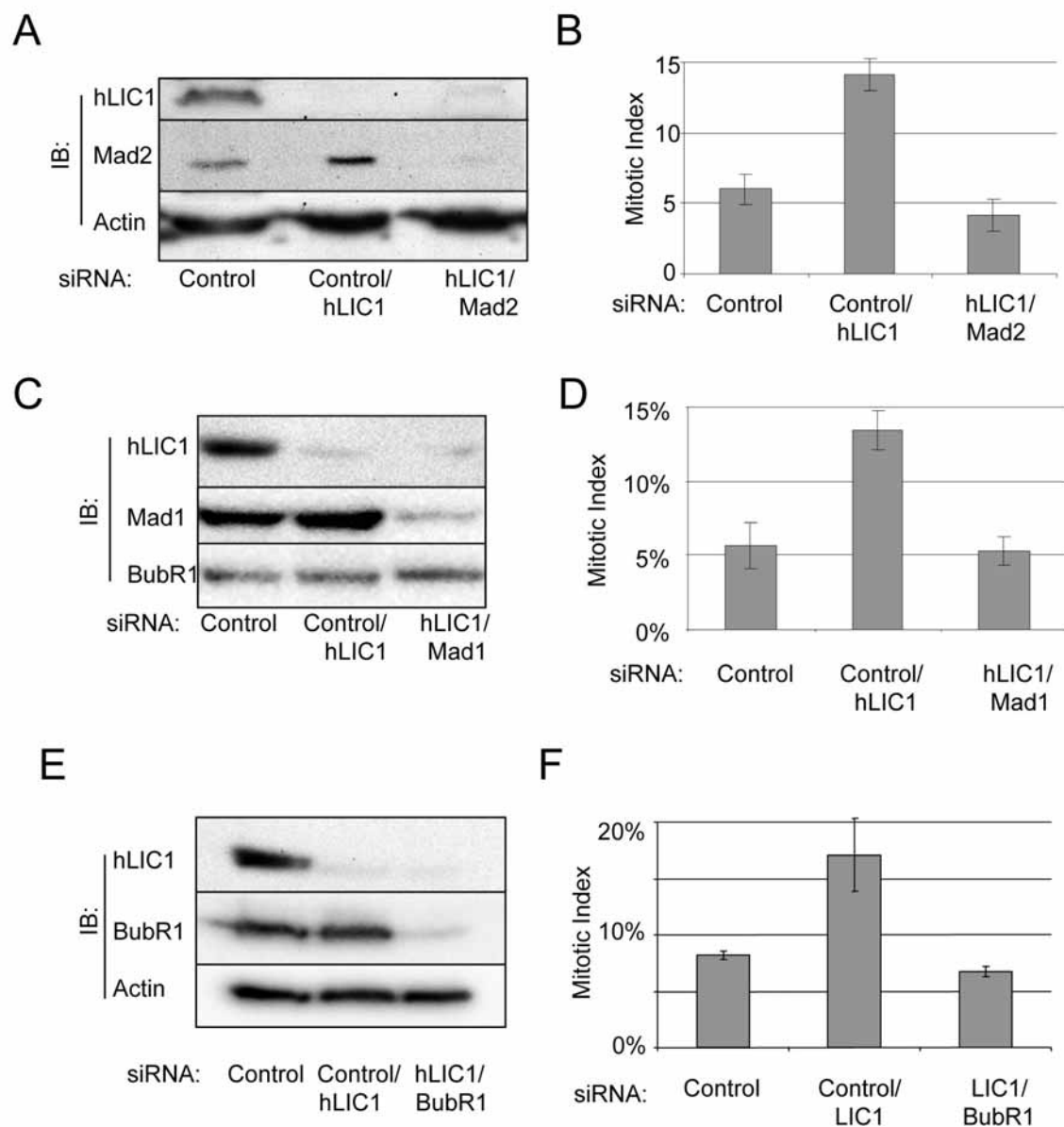


Figure 6. Mitotic arrest induced by LIC1 depletion requires Mad2, Mad1, and BubR1

A. Immunoblots showing hMad2 and hLIC1 following treatment of siRNAs: control, control/hLIC1 or hLIC1/Mad2. **B.** Mitotic indexes \pm SD after indicated siRNA treatments (each bar represents 1200 cells from 3 experiments). **C.** Immunoblots showing hMad1 and hLIC1 following treatment of siRNAs: control, control/hLIC1 or hLIC1/Mad1. **D.**

Mitotic indexes \pm SD after indicated siRNA treatments (each bar represents 1200 cells from two experiments). **E.** Immunoblots showing BubR1 and hLIC1 following treatment of siRNAs: control, control/hLIC1 or hLIC1/BubR1. **F.** Mitotic indexes \pm SD after indicated siRNA treatments (each bar represents 900 cells from three experiments).

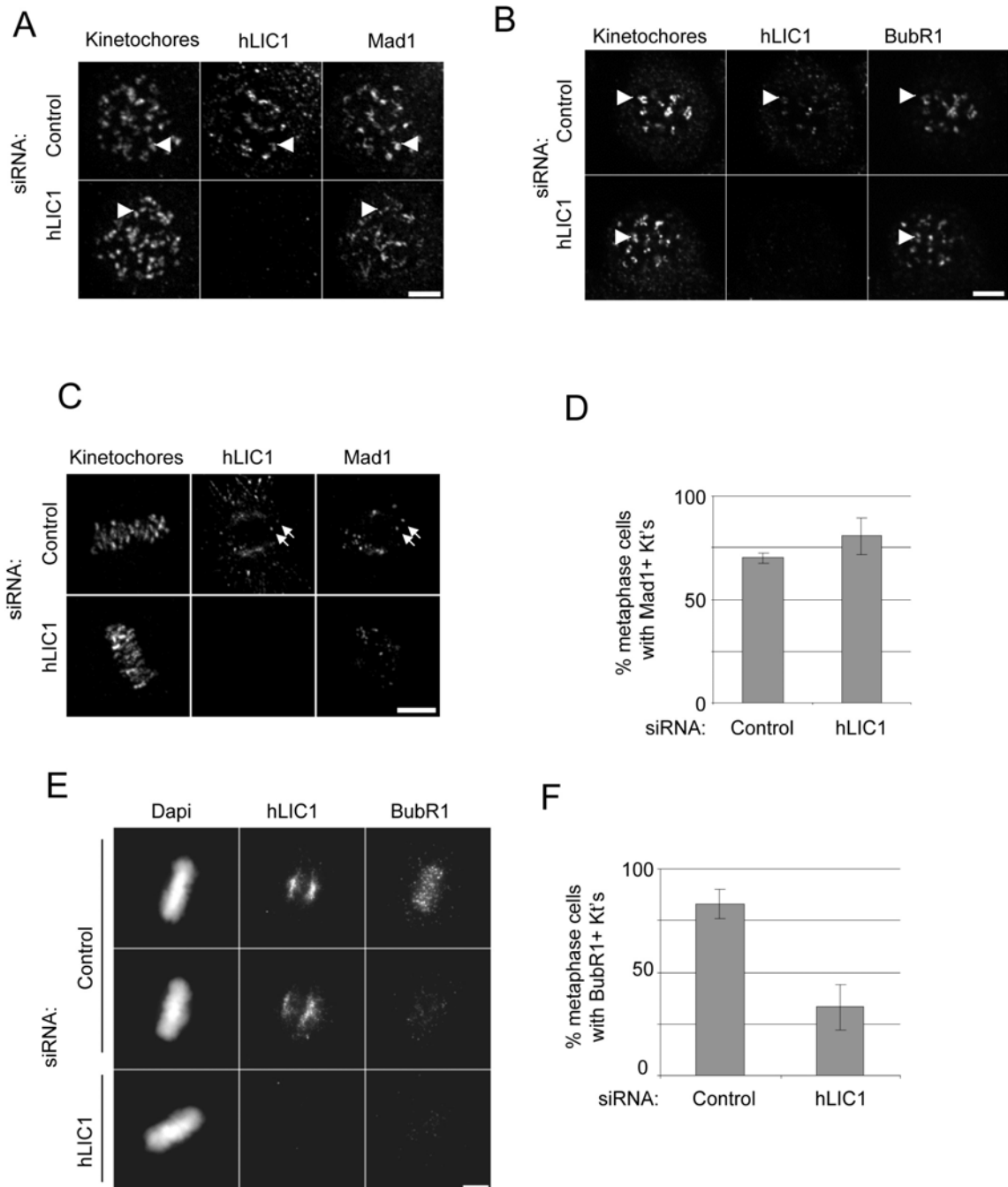


Figure 7. Mad1 and BubR1 localize to kinetochores in LIC1 depleted nocodazole treated cells but only Mad1 is inefficiently removed from metaphase kinetochores.

A. Mad1 localizes to kinetochores in nocodazole treated cells depleted of hLIC1 just as in control (GFP siRNAs). Pictures are a single plane image. Arrow heads point to a typical kinetochore. Bars in A, B, C, E, 5 μ m. Kinetochore, CREST stain. **B.** BubR1 localizes to kinetochores in nocodazole treated cells depleted of hLIC1 just as in control. Pictures are maximum projections of 3 planes 0.3 microns apart. Arrow heads point to a typical kinetochore. **C.** Metaphase cell showing Mad1 at kinetochores following the indicated siRNA treatments. Arrows show colocalization of hLIC1 and Mad1 when Mad1 is present on kinetochores. **D.** The average percentage \pm SD of metaphase cells that have one or more kinetochores positive for Mad1 (Mad1+, n=100 cells in two experiments for each group). Kt's, kinetochores. **E.** Metaphase cells showing BubR1 staining following the indicated siRNA treatments. DNA, DAPI stain. **F.** The average percentage \pm the standard deviation of metaphase cells that have one or more kinetochores positive for BubR1 (control, n=100 cells from two experiments, LIC1, n=150 cells from three experiments).

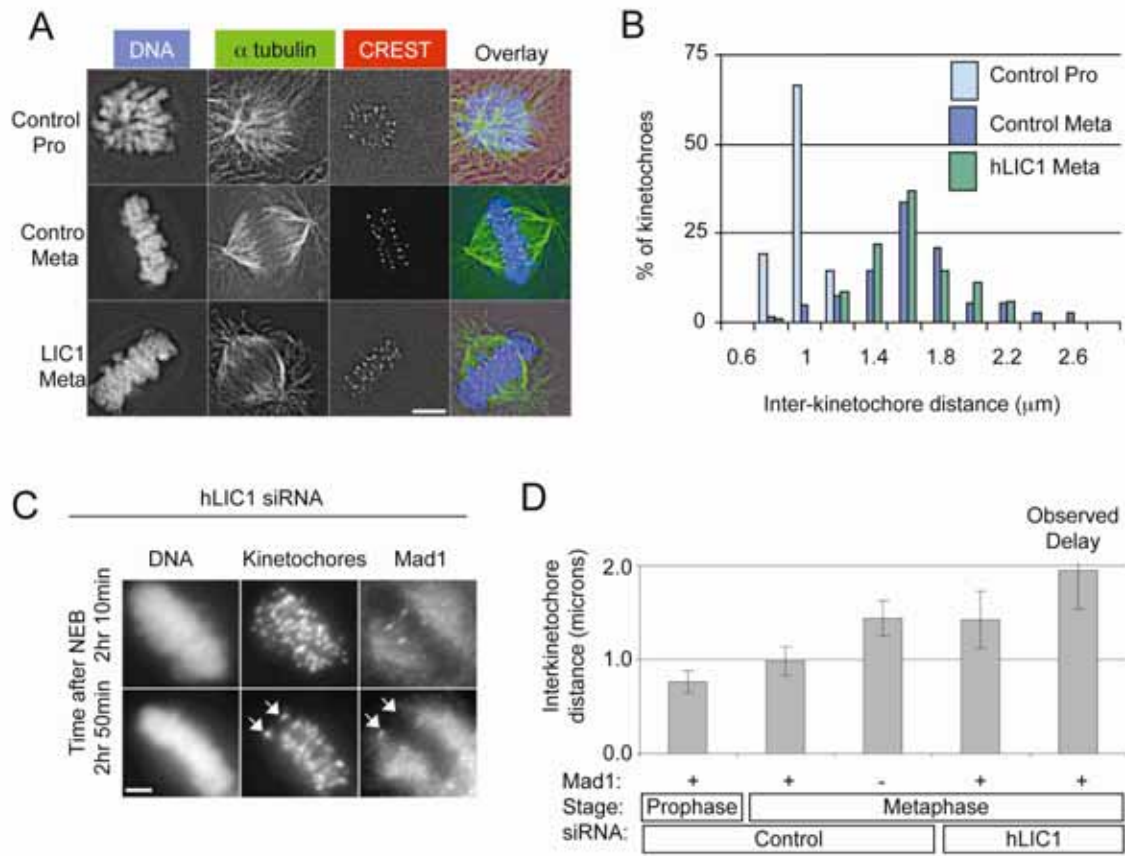


Figure 8. Mad1 remains at a few kinetochores that are under tension in most LIC1-depleted mitotic-delayed cells

A,B. Images (A) and quantification (B) of inter-kinetochore distances in prophase (pro) cell or metaphase (meta) SAOS cells treated with the indicated siRNA and labeled as indicated. Pictures are maximum projections of 2 planes 0.2 microns apart. Similar results were observed in HeLa cells. Average inter-kinetochore distance \pm SD in SAOS and HeLa cells respectively are 0.9 ± 0.1 microns ($n=21$ pairs from 1 cell) and 0.8 ± 0.1 microns ($n=47$ pairs from 2 cells) in control prophase cells, 1.5 ± 0.4 microns ($n=119$ from 10 cells) and 1.6 ± 0.2 microns ($n=76$ from 3 cells) in control metaphase cells, and 1.5 ± 0.3 ($n=105$ pairs from 9 cells) and 1.6 ± 0.3 ($n=97$ pairs from 4 cells) in hLIC1

depleted metaphase cells. **C.** Images showing two hLIC1 siRNA treated cells stained for Mad1 at least two hours after NEB, as determined by time lapse imaging to show tension on Mad1 positive kinetochores. Top panels show a cell with three Mad1 positive kinetochores and the bottom panels show a pair of sister kinetochores (arrows) that are under tension and have Mad1 localized to one of them. Pictures are maximum projections of 2 planes 0.25 microns apart. **D.** Graph shows average distances \pm SD between sister kinetochore pairs for the following groups: control prophase kinetochores positive for Mad1 (n=18 pairs from 1 cell), control metaphase kinetochores positive for Mad1 (n=16 pairs from 7 cells), control metaphase kinetochores negative for Mad1 (n=82 pairs from 10 cells), hLIC1 treated metaphase kinetochores positive for Mad1 in a randomly cycling population (n=26 from 11 cells) and Mad1 positive kinetochores in hLIC1 treated metaphase cells observed to be delayed in mitosis for more than 100 minutes (n=5 from 4 cells).

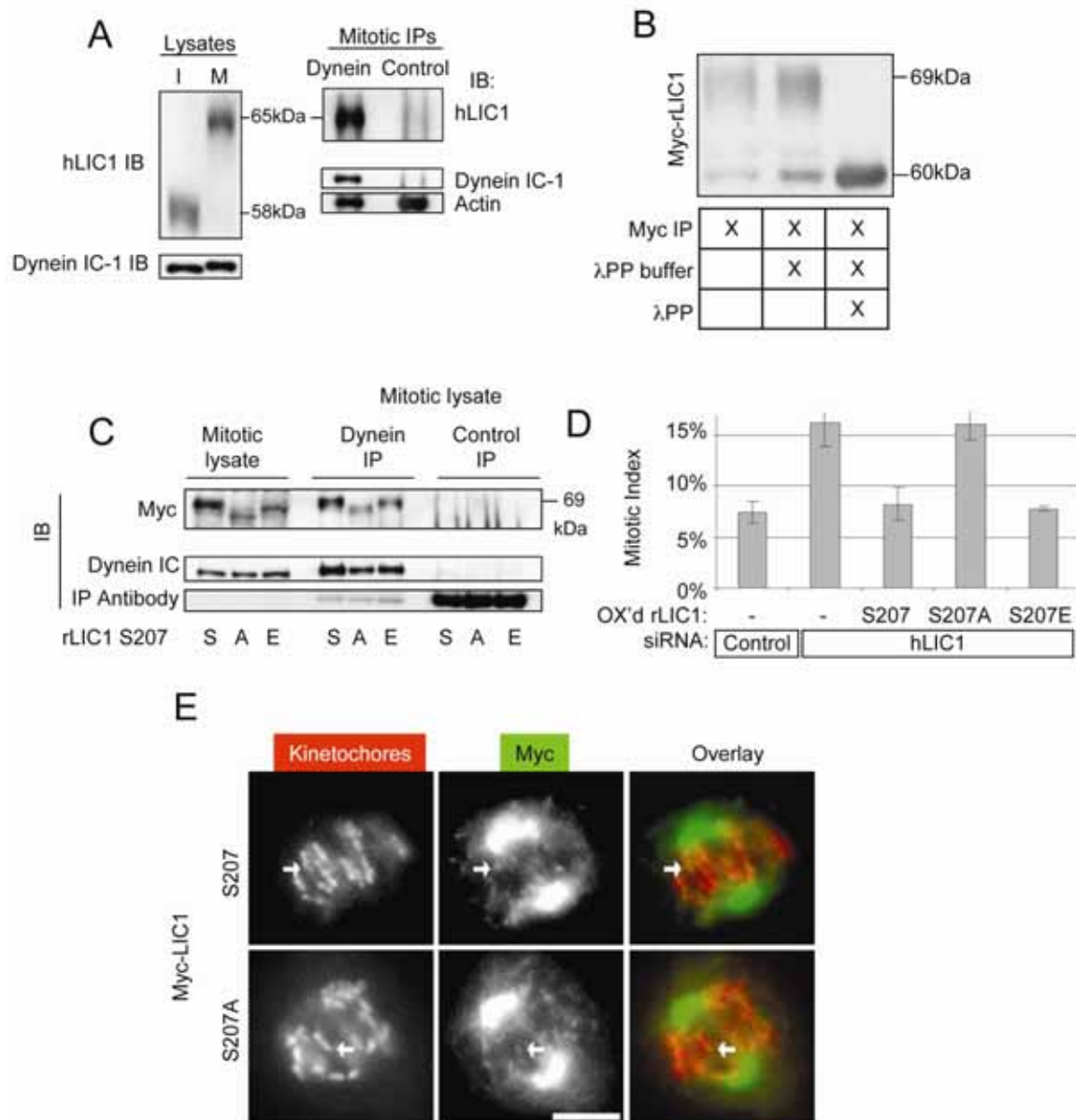


Figure 9. Phosphorylation of rLIC1 on S207 is important for resolution of the spindle assembly checkpoint

A. Endogenous hLIC1 in interphase cells undergoes a gel shift during mitosis. The mitotic form of LIC1 co-immunoprecipitates with dynein IC-1 from mitotic lysates. I, interphase; M, metaphase. **B.** A myc-tagged rLIC1 also undergoes a mitotic shift and is

reduced to the lower form with lambda protein phosphatase treatment (λ PP). **C.** The mitotic shift of myc-rLIC1 can be reduced by the S207A mutation and restored with a S207E point mutation (three left lanes). All myc-rLIC1 mutants co-immunoprecipitate with dynein IC-1 antibodies (center) but not control IgG (right). S, A, E represent S207, S207A, S207E respectively. **D.** Mitotic index \pm SD of HeLa cells treated with control or hLIC1 siRNA and overexpressing myc-rLIC1 or point mutants of this construct as indicated (each bar, 1200 cells from two experiments, except S207E, 600 cells from one experiment). OX'd, overexpressed. **E.** Immunofluorescence of myc-rLIC1 wild type or the S207A mutant with CREST staining of kinetochores. Arrows show myc tagged constructs co-localizing with kinetochores.

Discussion

A novel role for LIC1 in the spindle assembly checkpoint

Spindle assembly checkpoint control by LIC identifies a new role for this dynein subunit. An earlier study showed that LIC1 phosphorylation in mitosis drove the complex off vesicles, but the mitotic function was not examined (Addinall et al., 2001). Another study demonstrated that the solitary LIC in *Caenorhabditis elegans* was required for mitosis, but the mitotic stage affected by LIC disruption was not identified and the mechanism of LIC function was not addressed (Yoder and Han, 2001). This study also showed that functional abrogation of the *Caenorhabditis elegans* LIC prevented centrosome separation and induced monopolar spindle formation, a phenotype not observed in this study. The additional phenotypes observed following mutation of the solitary LIC in *Caenorhabditis elegans* could result from loss of functions associated with both human LICs (1 and 2) or from differences in LIC function between embryonic and somatic cell systems. It is also possible that additional LIC1 functions are not revealed in this study due to residual levels of LIC1 that remain after siRNA depletion.

Our work suggests that LIC1 is directly involved in the spindle assembly checkpoint and is not simply a structural component of the kinetochore. The dependence of the LIC1 depletion-induced metaphase delay on Mad1, Mad2, and BubR1 shows that an active checkpoint is required but it does not rule out disruption of kinetochore organization as a contributing factor to the phenotype. However, localization of Mad1, BubR1 and dynein

to kinetochores in nocodazole-treated LIC1 depleted cells shows that LIC1 does not significantly perturb kinetochore structure. Moreover, localization of the Mad1-Mad2 complex, as implicated by Mad1 localization, to kinetochores in LIC1-depleted metaphase-delayed cells shows that the checkpoint-signaling platform is intact at the kinetochore. Previous studies showed that partial depletion of kinetochore components involved in kinetochore-microtubule attachment (Hec1 and Nuf2, Ndc80 complex components) induced defects in chromosome congression, compromised Mad2 recruitment to kinetochores in nocodazole treated cells and led to metaphase arrest (DeLuca et al., 2003; Martin-Lluesma et al., 2002; Meraldi et al., 2004). Importantly, more extensive depletion had the opposite defect – suppression of the checkpoint – consistent with complete loss of the checkpoint signal pathway from the kinetochore (e.g. Mad1-Mad2) (Meraldi et al., 2004). LIC1 does not appear to fall into this category of kinetochore proteins, as chromosome congression was normal in LIC1 depleted cells and checkpoint proteins were recruited normally in nocodazole treated cells. We conclude that LIC1 is involved in attenuation of the wait anaphase signal rather than structural organization of the kinetochore.

Why is the LIC1-depletion phenotype partially penetrant? LIC1 siRNA treatment induces a metaphase delay in about half the cells even though the protein levels are reduced to 70-90% of control levels. This could be explained by cell-to-cell variation in LIC1 levels, which has been observed (data not shown). It is also possible that LIC2 can compensate for LIC1 function and that near complete depletion of LIC1 is required to overcome LIC2 compensatory function. However, independent treatment of cells with two different

siRNAs specific for LIC2 had no detectable effect on progression through mitosis and did not exacerbate the LIC1 phenotype (unpublished data). Further investigation of LIC2 will be required to test its role in the spindle assembly checkpoint. However, LIC1 depletion by itself is enough to result in a wait anaphase signal showing the importance of this specific dynein subunit in the spindle assembly checkpoint.

Mad1-Mad2 localization to kinetochores is sufficient to produce wait anaphase signal

The observation that Mad1 remains on kinetochores that are under tension and have very little of the tension-associated protein BubR1 is novel. The spindle assembly checkpoint is normally not active when kinetochore-associated BubR1 is reduced to control levels. This is because Mad1-Mad2 is typically removed early, after microtubules are attached to kinetochores, whereas BubR1 is removed later, after sister kinetochores are under tension (Shannon et al., 2002; Skoufias et al., 2001; Taylor et al., 2001). Our results suggest that kinetochore-associated Mad1-Mad2 alone is sufficient to activate or maintain the spindle assembly checkpoint. The need for BubR1 for LIC1-depletion delay is consistent with other spindle assembly checkpoint related mitotic delays where Mad1-Mad2 are localized to kinetochores (Meraldi et al., 2004). The need for BubR1 in the LIC1-depletion delay where it is only present at low levels on kinetochores suggests a cytoplasmic BubR1 function in checkpoint activation, possibly in propagation of the signal. BubR1 has been found to be in a complex with Mad2 and Cdc20 lending further support to a cytoplasmic role for BubR1 (Sudakin et al., 2001). Thus, the LIC1-depletion delay demonstrates that

kinetochore Mad2 is sufficient to produce a wait anaphase signal that needs cytoplasmic BubR1 and it also provides a novel system to study the role of cytoplasmic BubR1 in producing a wait anaphase signal.

A model for LIC1 function: Adaptor for dynein cargoes at the kinetochore

The role of LIC1 in the spindle assembly checkpoint represents one of many mitotic functions performed by cytoplasmic dynein (Gaglio et al., 1997; Heald et al., 1997; Howell et al., 2001; Wojcik et al., 2001). The role of dynein in the spindle assembly checkpoint appears to involve movement of spindle assembly checkpoint proteins such as Mad1-Mad2, BubR1 and Zw10 from kinetochores to spindle poles (Howell et al., 2001; Wojcik et al., 2001). In LIC1 depleted cells, BubR1 removal from kinetochores occurs normally, consistent with the normal localization and function of dynein. In contrast, Mad1 is inefficiently moved off kinetochores. This suggests that Mad1-Mad2 but not BubR1 is specifically uncoupled from the motor in the absence of LIC1. Our data are consistent with a model in which LIC1 serves as an adaptor linking specific cargoes to the dynein motor for their removal from kinetochores and resolution of the spindle assembly checkpoint.

Mad1-Mad2 is not the only cargo linked to dynein by LIC1. The centrosome protein pericentrin binds directly to LIC1 (Purohit et al., 1999) and is transported to centrosomes by cytoplasmic dynein (Young et al., 2000). LIC1 also interacts with Rab4A, a GTPase

involved in the regulation of intracellular vesicle trafficking (Bielli et al., 2001). Because the interaction of dynein with interphase membranes/vesicles is regulated by mitotic phosphorylation (Addinall et al., 2001), it is possible that mitotic phosphorylation of LIC1 down regulates its affinity for Rab4A and other interphase vesicle proteins to allow targeting of mitotic cargoes. The precise role of LIC1 in the transport of vesicles and proteins in interphase and mitosis is currently under investigation.

The role of LIC1 in binding dynein cargoes is consistent with similar roles of other dynein and dynactin subunits, which bind NuMA, PCM-1, spectrin, rhodopsin and IFT proteins (Hou et al., 2004; Pfister, 2005; Zimmerman and Doxsey, 2000). In fact, other dynein subunits may be involved in regulating Mad1-Mad2 removal from kinetochores as depletion of the Rab6A' GTPase, a potential regulator of dynein/dynactin function through the dynactin complex, results in Mad1-Mad2 remaining at a few kinetochores and an active spindle assembly checkpoint (Miserey-Lenkei et al., 2006). The use of dynein and dynactin subunits to link and regulate the binding of multiple cargoes to the minus end dynein motor (Pfister et al., 2006) appears to provide a mechanism analogous to amplification of the kinesin gene family, which links cargoes to multiple different kinesins (Goldstein, 2001).

A model for Mad1-Mad2 localization to just a few kinetochores in LIC1 depletion

Our data from LIC1 depleted cells suggest that Mad1-Mad2 bound to a subset of kinetochores can produce the wait anaphase signal that prevents anaphase onset. It has also been suggested that checkpoint signaling occurs from the few Mad2-positive kinetochores in taxol treated cells leading the authors to propose that Mad1-Mad2 will localize to those kinetochores with weak kinetochore-microtubule attachments (i.e. fewer microtubules occupy the kinetochore) under these conditions (Waters et al., 1998). In the case of LIC1 depletion, we propose that kinetochore-microtubule attachments are transiently lost when kinetochores are under tension. In normal cells, Mad1-Mad2 would be recruited to these detached kinetochores then moved off as kinetochore-microtubule attachments are re-established. Normally, these events would go unnoticed because Mad1-Mad2 removal from kinetochores would be rapid and efficient. However, in LIC1 depleted cells Mad1-Mad2 would be retained on kinetochores for longer periods of time due to inefficient removal by dynein. During this delay in Mad1-Mad2 removal, kinetochore-microtubule connections could be lost on other chromosomes and accompanied by Mad1-Mad2 recruitment to these new sites. This cycle of events could occur repeatedly in cells impaired for kinetochore removal of Mad1-Mad2 and could explain the metaphase delay and Mad1-Mad2 localization to a few kinetochores.

This model is supported by the observation that merotelic attachments (one kinetochore attached to two poles) are resolved when they are in proximity to the inter-kinetochore

region of the chromosome, where MCAK, ICIS and Aurora B are thought to destabilize microtubules (Andrews et al., 2004; Kline-Smith et al., 2004; Ohi et al., 2003). It is possible that a similar mechanism could occasionally destabilize kinetochore microtubules when they are in proximity to an inter-kinetochore region of a neighboring chromosome on the metaphase plate where chromosomes are tightly packed (Mitchison and Salmon, 1992; Skibbens et al., 1993; Waters et al., 1996). This idea is also supported by observations showing that inter-kinetochore distances between normally bi-oriented chromosomes are sometimes reduced to resting distances (6.4%), suggesting that there is little to no tension across sister kinetochores (Waters et al., 1996). The authors state that this phenotype is a consequence of dynamic spindle microtubules pushing kinetochores together. On the other hand, it is also possible that kinetochore-microtubule attachments are broken due to microtubule-depolymerization activity contained in inter-kinetochore regions of adjacent chromosomes. This allows for infrequent breaks or at least severe impairments of kinetochore-microtubule attachments, that would be enough to keep Mad1-Mad2 localized to a few kinetochores, when its removal is impaired by LIC1 depletion.

LIC1 phosphorylation is required for its role in the spindle assembly checkpoint

In addition to providing a novel function for LIC1, our work extends the observation that mitotic phosphorylation of dynein by Cdk1 disengages the motor from interphase membrane cargoes (Addinall et al., 2001), thus demonstrating a role for phosphorylation

at the S207 Cdk1 site in the spindle assembly checkpoint. Phosphorylation would presumably promote the interaction of LIC1 with a cargo, which has yet to be identified. The obvious candidate cargo is Mad1-Mad2 as it remains on kinetochores in LIC1 depleted cells. However, neither Mad1 nor Mad2 interacts directly with LIC1 or LIC1 phosphorylation mutants *in vitro* (unpublished data). Despite this, the phosphorylation mutants will provide powerful tools to identify LIC1 interacting proteins and to determine the molecular mechanism by which LIC1 functions in the spindle assembly checkpoint.

We believe that Cdk1 is the kinase that phosphorylates LIC1 at S207 during mitosis because cyclin B accumulates and activates Cdk1 specifically in mitosis. Moreover, Cdk1 phosphorylates S207 *in vitro* (Dell et al., 2000) and this site is phosphorylated during mitosis (this study). In addition, other kinases with similar consensus sites for phosphorylation (e.g. Cdk2 and MAPK) are not good candidates for S207 phosphorylation. The Cdk2 activators cyclin A and cyclin E are both low in cells arrested in mitosis via the spindle assembly checkpoint (D'Angiolella et al., 2003). MAPK is also not active under normal conditions or when the spindle assembly checkpoint is active (Deacon et al., 2003). Thus, the probable candidate for phosphorylation of LIC1 at S207 is Cdk1.

It is interesting to note that inhibition of Cdk1 promotes entry into anaphase (D'Angiolella et al., 2003; Potapova et al., 2006). This is in contrast to re-expression of the non-phosphorylatable rLIC1 mutant, which does not promote entry into anaphase in

LIC1 depleted cells. One explanation for this difference is the observation that Cdk1 appears to have two antagonistic roles in mitosis. Cdk1 activity inhibits anaphase onset but it also sets up an environment that leads to its own inactivation by cyclin B1 degradation and thus promotes anaphase onset (D'Angiolella et al., 2003). These two roles of Cdk1 insure that chromosomes are attached and under enough tension for the spindle assembly checkpoint to be inactivated before cells transition from metaphase to anaphase. This also means that inhibitors of Cdk1 will allow cells into anaphase disregarding information from the spindle assembly checkpoint, which includes the function of phosphorylated LIC1.

Materials and methods

Cell Culture

HeLa cells (ATCC CCL-2) and SAOS (ATCC HTB-85) were cultured as described by American Type Cell Collection. SAOS cells were only used in figure 8A,B.

siRNA treatment

siRNAs (21-nt; Dharmacon Research, Inc.) targeting hLIC1 (GenBank/EMBL/DDBJ accession no. NM_016141; nt 1156-1174), GFP (Gromley et al., 2003), hMad2L1 (NM_002358; nt 503-523) and hMad1 (Martin-Lluesma et al., 2002) were delivered to cells using Dharmafect 1 (Dharmacon Research, Inc). hLIC1 siRNA target sequence is specific to hLIC1 and does not completely match rLIC1 or hLIC2.

Constructs and overexpression

C-terminal myc tagged full length rLIC1 (Tynan et al., 2000b) was used for Figure 4. The constructs in Figure 9 were made in the following manner: full length rLIC1 was generated by PCR from the C-terminal myc tagged full length rLIC1 vector (primers used: CCG GAA TTC ATG GCG GCC GTG GGG CGA GTC and CTG TGC CTC GAG TCA GGA GGC TTC TCC TTC CGT AGG), ligated into the pDNR-2 (Clontech Laboratories, Inc) using EcoRI and XhoI cut sites, and sequenced. Mutations were introduced into the rLIC1 sequence using the QuickChange site-directed mutagenesis protocol (Stratagene) and the following primers: TCC CAG CCG CCC CTC and GAG GGG CGG CTG GGA for S207A; TCC CAG CCG AAC CTC AGC G and CGC TGA GGT TCG GCT GGG A for S207E. Mutations were verified by sequencing. All three

constructs of rLIC1 were ligated into pLP-CMV-Myc as described by the manufacturer (Clontech Laboratories, Inc). Constructs were delivered to cells using Lipofectamine 2000 (Invitrogen).

LIC1 antibody production

A peptide consisting of the C-terminal 15 amino acids of hLIC1: VFP TTP TSP TEG EAS (Bielli et al., 2001) was synthesized. Polyclonal antibodies were produced against this peptide in rabbits by Q-biogene. Antibody was affinity purified on a peptide column of CNBr-activated sepharose 4B beads (Amersham) (Harlow and Lane, 1999).

Time-lapse imaging

HeLa cells were imaged as previously described (Gromley et al., 2003). In Figure 3B, cells while still on the stage were incubated in 0.3 μ M Syto13, a viable DNA stain (Invitrogen) for 5 minutes and then imaged.

Antibodies, Fixation, Staining and Imaging

Cells were fixed in methanol, or formaldehyde (Dictenberg et al., 1998). For staining Mad2, cells were permeabilized and fixed in formaldehyde as previously described (Hoffman et al., 2001). The following antibodies were used: Anti dynein intermediate chain 74.1, anti cyclin B1, and anti α -tubulin DM1 α culture supernatant (Sigma); anti hMad1 (De Antoni et al., 2005); anti LIC1 (described above); and CREST serum (Brenner et al., 1981). Golgi was directly stained with *Helix pomatia* agglutinin Alexa 488 (Invitrogen). Images were either taken on a wide field microscope as previously described (Dictenberg et al., 1998) or on a confocal microscope: Zeiss Axiovert 200M, 100x Plan-APOCROMAT NA1.4 Oil, DIC lens, and Hamamatsu ORCA-ER camera. The

entire fixed cell volume was imaged and displayed as a two-dimensional projection (Meta-Morph; Universal Imaging Corp.). Cyclin B1 levels were measured by integrating the fluorescence intensity of the whole cell. To look at Mad1 and BubR1 localization to unattached kinetochores, cells were treated with 1 μ M nocodazole for 1 hour to depolymerize microtubules before fixation. The images for DNA and α -tubulin in figure 8A were run through a 2D-deconvolution algorithm (Meta-Morph; Universal Imaging Corp.).

Western Blotting

Proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with the following antibodies: Anti dynein intermediate chain 74.1 and anti actin AC40 (Sigma); anti dynein heavy chain R-325 and anti myc 9E10 (Santa Cruz); anti hMad1 (De Antoni et al., 2005); anti hMad2 (PRB-452C Covance); and anti LIC1 (described above). Anti-rabbit and anti-mouse HRP antibodies were used along with ECL plus luminescent reagent (Amersham Biosciences) and then visualized on film or using a 4000MM Image Station (Kodak).

Immunoprecipitation and Phosphatase treatment

Lipofectamine 2000 (Invitrogen) was used to transfect cells following the manual protocol. At 24 hours, 1 μ g/ml nocodazole was added. At 48 hours, mitotic cells were collected by pipetting PBS over the plate several times. Cells were lysed in 20mM Tris HCl pH 7.5, 50mM NaCl, 1mM EGTA, 1% Triton X-100, 50mM NaF, 10mM B-Glycerophosphate, 5mM Na₄P₂O₇, 1mM Na₃VO₄, 1 μ M microcystin.

Immunoprecipitations were performed with myc or 74.1 dynein intermediate chain

antibodies and Protein G Plus-Agarose beads (Santa Cruz Biotechnology). For phosphatase treatment, beads were treated in one of three ways: 2x SDS buffer was added; or 30 minutes incubation at 30°C in Lambda protein phosphatase buffer and then 2x SDS buffer was added; or 30 minutes incubation at 30°C in Lambda protein phosphatase buffer and Lambda protein phosphatase (New England Biolabs Inc.) and then 2x SDS buffer was added.

Sucrose gradient

Cells were harvested with 10mM EDTA in PBS. Cells were lysed in 50mM Tris-Cl pH 7.5, 150mM NaCl, 1% IGEPAL CA-630, 1mM EDTA, 1 complete protease inhibitor cocktail mini tablet (Roche) for each 10ml of lysis buffer. Lysates were spun at 16,000 RCF at 4°C for 10 minutes to pellet cellular debris. The supernatant was layered on a 12ml 5-20% sucrose gradient, and centrifuged at 4°C in a Beckman Coulter Optima L-90K ultracentrifuge (35,000 rpm, SW41 rotor) for 12.5 hours. Sedimentation standards used were thyroglobulin 19.4S, catalase (11.3S), aldolase (7.3S) and BSA (4.4S). Sucrose gradient fractions (500ul) were collected and analyzed by western blots.

Chapter 3: Light intermediate chain 1 of cytoplasmic dynein is involved in pericentrin assembly

Introduction

The centrosome is a small justa-nuclear organelle made up of two centrioles around which there is a dense, partially filamentous matrix called the pericentriolar material or PCM (Brinkley, 1985; Doxsey, 2001). The PCM is involved in both nucleating and anchoring microtubules, allowing for the focal organization of microtubule minus ends at the centrosome in interphase cells. Pericentrin is a large coiled-coil protein and is a component of the PCM (Doxsey et al., 1994). Pericentrin appears to act as a scaffold at the centrosome both structurally (Dictenberg et al., 1998) and molecularly to possibly increase signaling or reaction efficiencies by localizing kinases and other proteins close to one another. Structurally, pericentrin forms a very intricate lattice network at the centrosome along with other proteins (Dictenberg et al., 1998). Molecularly, pericentrin appears to have several roles. First, pericentrin is part of a microtubule nucleating complex along with gamma-tubulin which is recruited to the centrosome through the cell

cycle so that greater nucleating capacity is possible in mitosis (Dictenberg et al., 1998; Kellogg et al., 1994; Kuriyama and Borisy, 1981; Young et al., 2000). Second, pericentrin is also a docking site for protein kinase C and cAMP dependent kinases, thus facilitating the signaling of these pathways at the centrosome (Chen et al., 2004; Diviani et al., 2000). Pericentrin appears to be an integral centrosome protein as it has an influence on many of the general functions of the centrosome from microtubule nucleation and organization to facilitating signaling networks at the centrosome.

The assembly of pericentrin to the centrosome therefore influences a number of different factors in the cell and if not controlled could lead to disruption or deregulation of signaling pathways and microtubule organization, especially important for mitotic spindle organization and genome stability (Chen et al., 2004; Pihan et al., 2001; Purohit et al., 1999). Pericentrin was found to assemble onto the centrosome through the cell cycle, starting from low levels in G1 and reaching maximal levels in metaphase. This assembly is followed by a disassembly in anaphase and telophase to reach low levels again in early G1 (Dictenberg et al., 1998). Pericentrin was found to interact with the molecular motor dynein and also move at dynein like speeds (Purohit et al., 1999; Tynan et al., 2000b; Young et al., 2000). Disruption of microtubules or the dynein motor complex resulted in pericentrin being unable to assemble to the centrosome (Young et al., 2000), implicating movement of pericentrin by dynein along microtubules in the assembly of pericentrin to the centrosome.

Dynein is a multi-protein motor complex. It has many functions within interphase from positioning of organelles, such as the endoplasmic reticulum, Golgi apparatus and nucleus, to the minus end directed transport of vesicles, such as endosomes, lysosomes, and melanosomes (Pfister, 2005; Pfister et al., 2006; Reilein et al., 2003). These numerous functions need to be regulated more or less independently from one another and so one motor seems hardly the correct choice for these multiple tasks. The dynein complex is made up of several subunits: heavy chains, intermediate chains, light intermediate chains and light chains. Each of these subunits has at least two highly homologous but different family members that influence the function of the dynein complex (Pazour et al., 1999; Tai et al., 2001; Tynan et al., 2000a; Tynan et al., 2000b). With the combinatorial association of subunits, there are many different subclasses of dynein that are important for different functions within the cell.

Pericentrin interacts specifically with dynein 1 light intermediate chain 1 (LIC1) of dynein and it is this interaction that is believed to be involved in pericentrin assembly onto the centrosome. The LICs do not appear to play a role in dynein motility but they have been shown to be important for attaching cargoes that need to be moved by dynein. (Hou et al., 2004; Reilein et al., 2003; Tynan et al., 2000a; Tynan et al., 2000b). Here we show that LIC1 is important for the assembly of pericentrin to the centrosome.

Results

Overexpression of the c-terminus of rLIC1 disrupts the assembly of pericentrin

In this study we used the c-terminus of rLIC1 to further understand the functional importance of the rLIC1-pericentrin interaction. We first confirmed the interaction of the c-terminus of rLIC1 with pericentrin using co-overexpression immunoprecipitation of full length pericentrin with the c-terminus of rLIC1 as well as the immunoprecipitation of the c-terminus of rLIC1 with pericentrin (figure 1).

Pericentrin assembles at the centrosome from low levels in early G1 to its highest levels in mitosis (Dictenberg et al., 1998). Thus the best point to start an assay for pericentrin assembly is with cells just after entry into G1. In our experiments cells were synchronized with thymidine and microinjected with cDNA for the c-terminus of rLIC1 in late G2, so that there would be a sufficient amount present to potentially interfere with endogenous LIC1-pericentrin interactions from early G1 till late G2. This is very similar to the assay that Young et al (2000) used to measure pericentrin assembly previously. Using the assay described above, we found that net pericentrin assembly was affected with the overexpression of the c-terminus of rLIC1 (figure 2).

The c-terminus of rLIC1 does not disrupt the microtubule cytoskeleton or dynein complex integrity or function

Pericentrin assembly to the centrosome through the cell cycle has previously been shown to require microtubules and a functional dynein complex (Young et al., 2000). We show here that the overexpression of the c-terminus of rLIC1 during one cell cycle does not affect these factors.

Overexpressing the c-terminus of rLIC1 during one cell cycle does not affect the microtubule cytoskeleton (figure 3). The dynein motor is also unaffected. First, it does not appear that dynein is degraded when the c-terminus of rLIC1 is overexpressed as the level of dynein intermediate chain 1, an integral component of the motor, does not change (figure 4A). Second, the dynein complex is intact when the c-terminus of rLIC1 is overexpressed as the dynein complex does not run differently in a sucrose gradient (figure 4B). An interesting point here is that the c-terminus of rLIC1 runs very high in the sucrose gradient, apart from dynein, showing that it is not incorporated with the dynein complex. This further supports the dominant negative effect of the c-terminus of rLIC1 because it can bind pericentrin but then does not associate with the dynein complex, not allowing the bound pericentrin to interact with the motor. Finally, overexpression of the c-terminus of rLIC1 does not affect the interphase localization of dynein to the centrosome or the ability of dynein to focus the Golgi apparatus (figure 4C-F). To conclude, the overexpression of the c-terminus of rLIC1 does not disrupt the microtubule cytoskeleton, nor does it interfere with dynein levels, integrity, interphase localization, or

interphase function, suggesting that pericentrin assembly should not be inhibited due to these factors.

Depletion of hLIC1 has some influence on the assembly of pericentrin

In an attempt to strengthen the idea that pericentrin assembles to the centrosome via LIC1 and dynein we looked at the assembly of pericentrin in cells depleted for LIC1 using RNAi. Using siRNA to target LIC1 we were able to deplete LIC1 protein levels by 70-86% at 24 to 48 hours after transfection of siRNAs in HeLa cells (figure 5A). To perform a synchronized pericentrin assembly assay in cells depleted for LIC1 we collected mitotic cells from a normally cycling population treated with control or LIC1 siRNA for 18 hours to try and avoid issues with mitotic delay in LIC1 depleted cells as described in chapter 2. Two hours after the mitotic shake off most all cells had divided and were in early G1. Measurements of total centrosomal pericentrin levels were taken at this early G1 time as a baseline to compare with cells that were allowed to grow and assemble pericentrin for 17 or 19 hours after the mitotic shake off. On average pericentrin did not assemble as much in LIC1 siRNA treated cells as compared to control (figure 5B), however as the depletion is not complete it appears that the low level of LIC1 protein that remains after LIC1 siRNA treatment is able to assemble pericentrin to near control levels (figure 6).

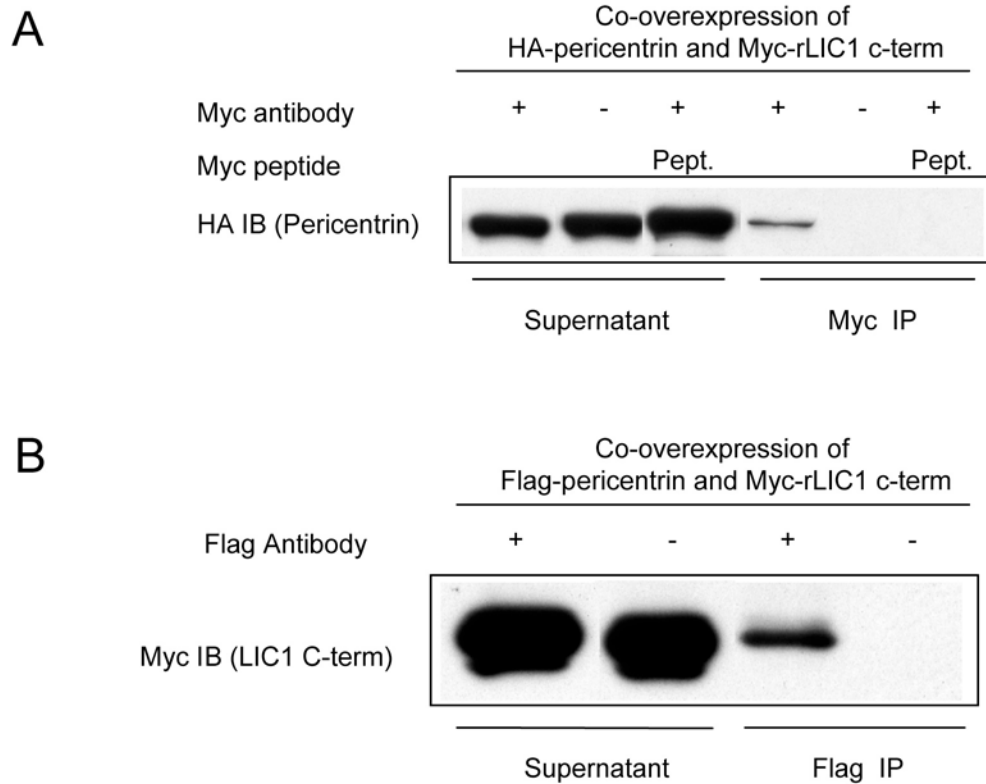


Figure 1: The c-terminus of rLIC1 and Pericentrin interact

A. Myc antibody immunoprecipitation (IP) of overexpressed myc tagged c-terminus of rLIC1 (Myc-rLIC1 c-term) pulls down overexpressed HA tagged pericentrin.

Immunoprecipitations were incubated with myc peptide (pept.) where mentioned to dissociate myc-rLIC1 from the myc antibody. Antibody was included in the reaction where indicated (+). IB, immunoblot. **B.** Flag antibody immunoprecipitation of

overexpressed flag tagged pericentrin pulls down overexpressed myc tagged rLIC1 c-terminal fragment.

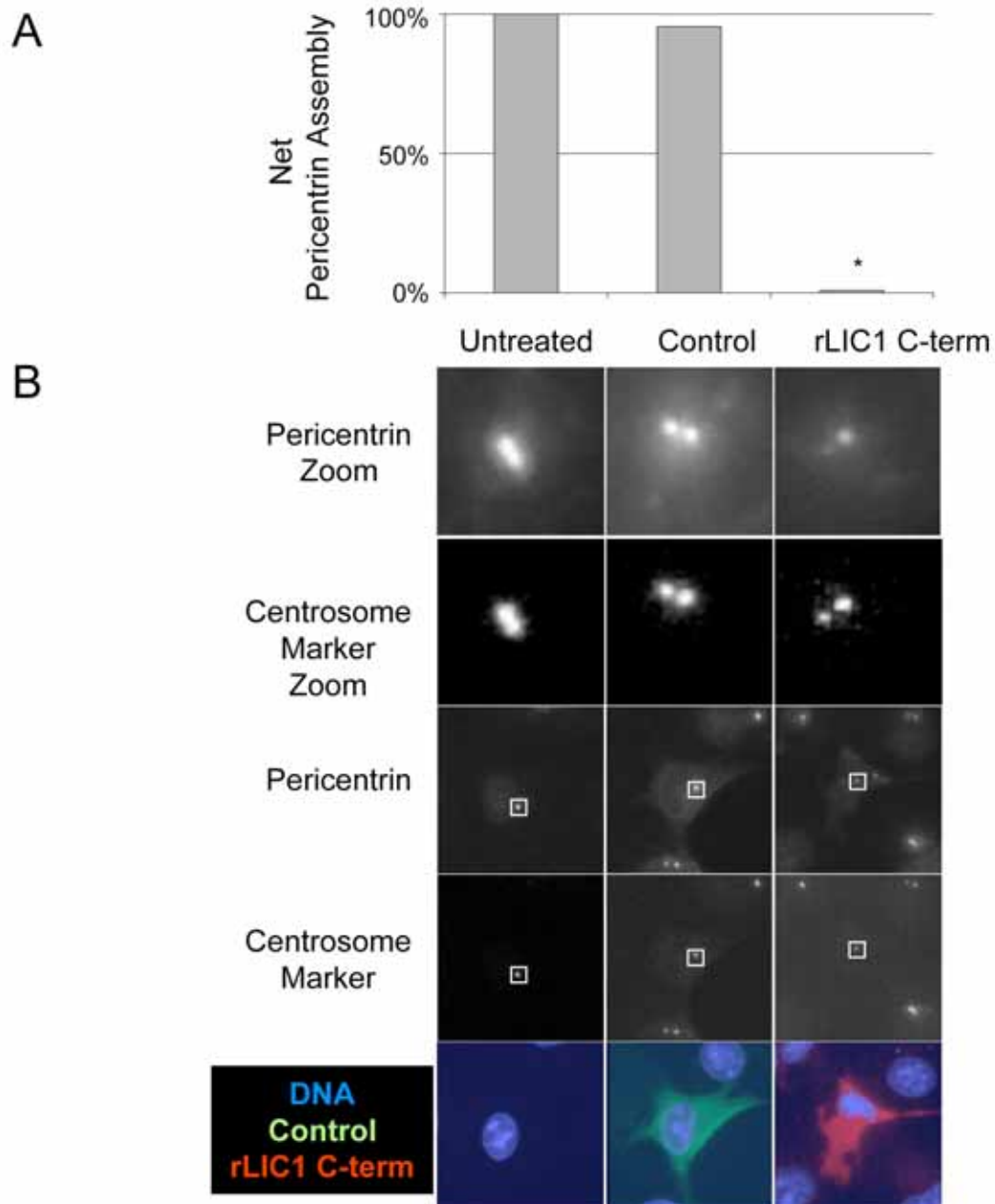
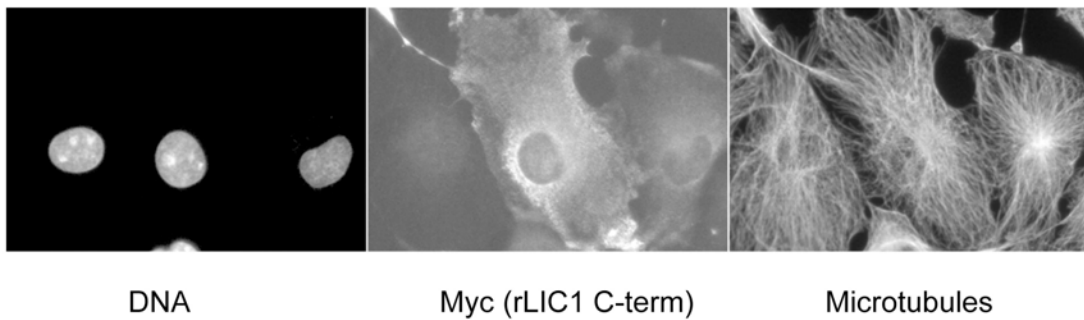


Figure 2: Overexpression of the c-terminus of rLIC1 disrupts the assembly of pericentrin

A. Net centrosomal pericentrin assembly in cells that were untreated, expressing a control construct or expressing the myc tagged c-terminus of rLIC1. Cells were microinjected so

that constructs would be expressing in early G1 and then were fixed in late G2 so that a near full pericentrin assembly period could be observed (n=24 cells for untreated, 81 for control and 24 for rLIC1 c-term). The graph shows averages of averages from different experiments. * $p < 0.0002$ compared to untreated. **B.** Immunofluorescence of pericentrin and a centrosomal marker (5051 serum) in untreated cells or cells overexpressing a control construct or the myc tagged c-terminus of rLIC1. Zoom indicates the expansion of the boxed area below.

A



B

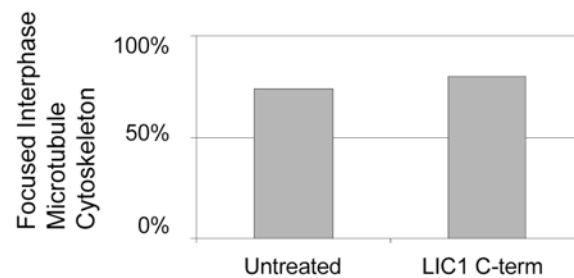


Figure 3: The c-terminus of rLIC1 does not disrupt interphase microtubule organization

A. Immunofluorescent images of focused microtubule cytoskeletons in two cells overexpressing myc tagged c-terminus of rLIC1 (center and right cells) and one that is not (left cell). DNA stained with DAPI. Microtubules stained with anti-alpha tubulin antibody. **B.** Percentage of cells with focused interphase microtubule cytoskeletons that are overexpressing a control construct or the myc tagged c-terminus of rLIC1 (n=38 for control, 15 for rLIC1 c-term).

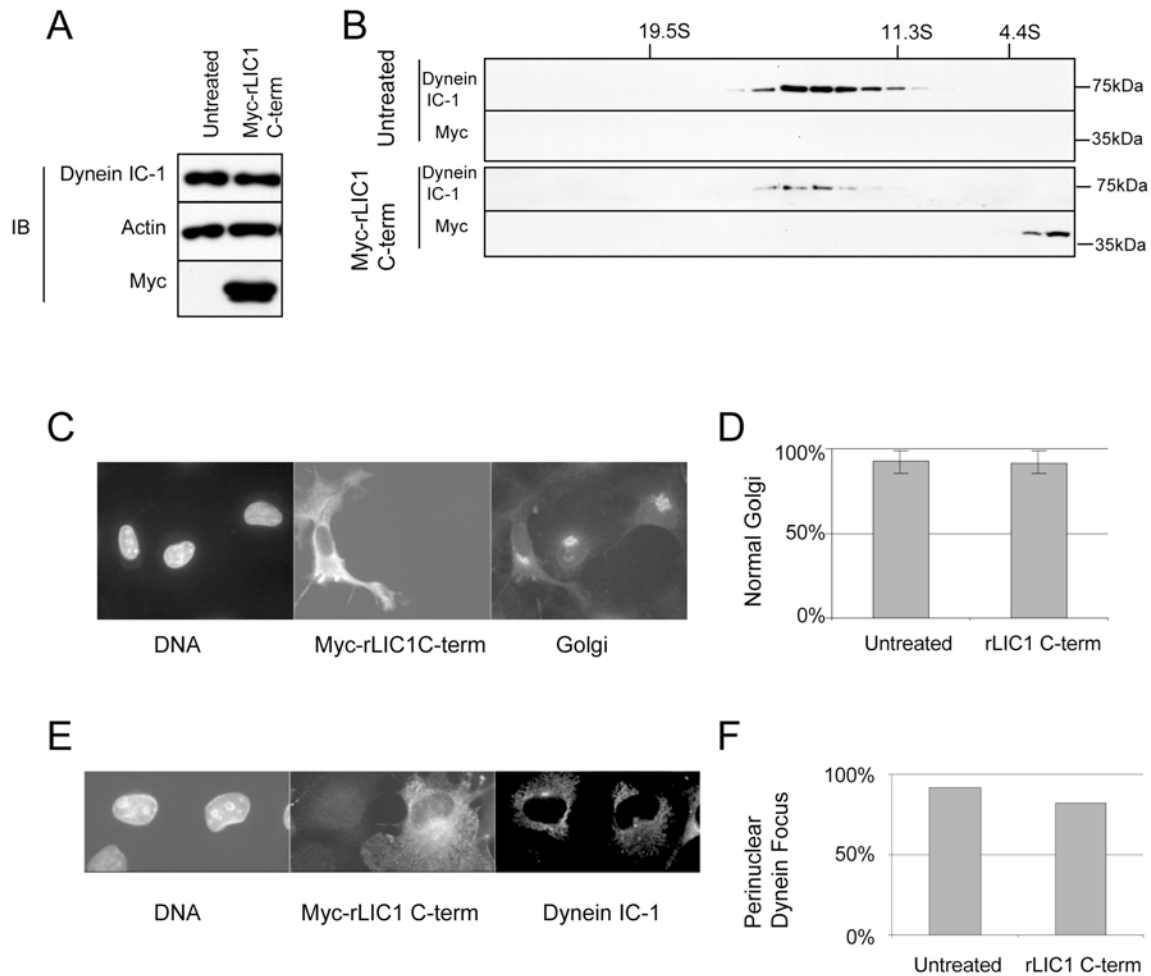


Figure 4: Overexpression of the c-terminus of rLIC1 does not disrupt dynein structure, localization or function.

A. Overexpression of myc tagged c-terminus of rLIC1 does not affect levels of dynein intermediate chain 1 (dynein IC-1), an integral component of dynein. **B.** Overexpressed myc tagged c-terminus of rLIC1 does not change the sedimentation of dynein intermediate chain 1. Also overexpressed myc tagged c-terminus of rLIC1 does not appear to associate with the dynein complex. S-value markers, above. The weak dynein IC-1 signal in the myc-rLIC1 c-term sucrose gradient compared to the control is due to a difference in the number of cells lysed for the experiment. **C.** Immunofluorescent images

of normal focused Golgi localization in one cell overexpressing myc tagged c-terminus of rLIC1 (left cell) and two cells that are not (center and right cells). **D.** Percentage of cells with normal focused Golgi localization that are overexpressing a control construct or myc tagged c-terminus of rLIC1 (n=63 over three experiments for control and 53 over 2 experiments for rLIC1 c-term). **E.** Immunofluorescent images of focal perinuclear dynein IC-1 staining in one cell overexpressing the myc tagged c-terminus of rLIC1 (right cell) and one cell that is not (left cell). **F.** Percentage of cells with a perinuclear focus of dynein IC-1 in cells overexpressing a control construct or the myc tagged c-terminus of rLIC1 (n=24 cells for control and 11 for rLIC1 c-term).

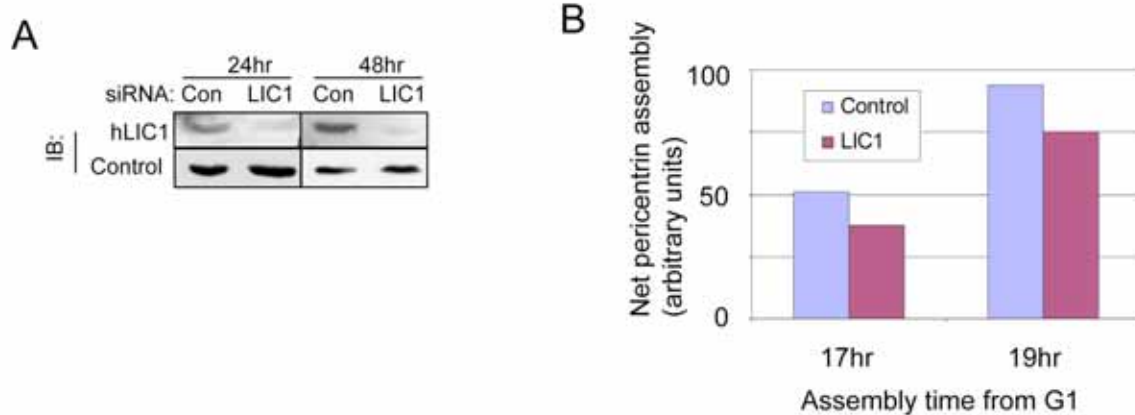


Figure 5: Depletion of hLIC1 disrupts pericentrin assembly to some degree

A. Immunoblot showing the depletion of hLIC1 in HeLa cells 24 and 48 hours after treatment with control (Con) or hLIC1 specific siRNA. Loading control for the 24 hour samples is actin and for the 48 hour samples is dynein intermediate chain 1. **B.** Graph shows the average net pericentrin assembly on the centrosome after a synchronized early G1 population of cells was allowed to freely cycle and assemble pericentrin for the times denoted. Early G1 cells were plated for this assay from a mitotic shake off of cells treated for 28 hours with control or LIC1 siRNA (n=11-13 cells for each bar).

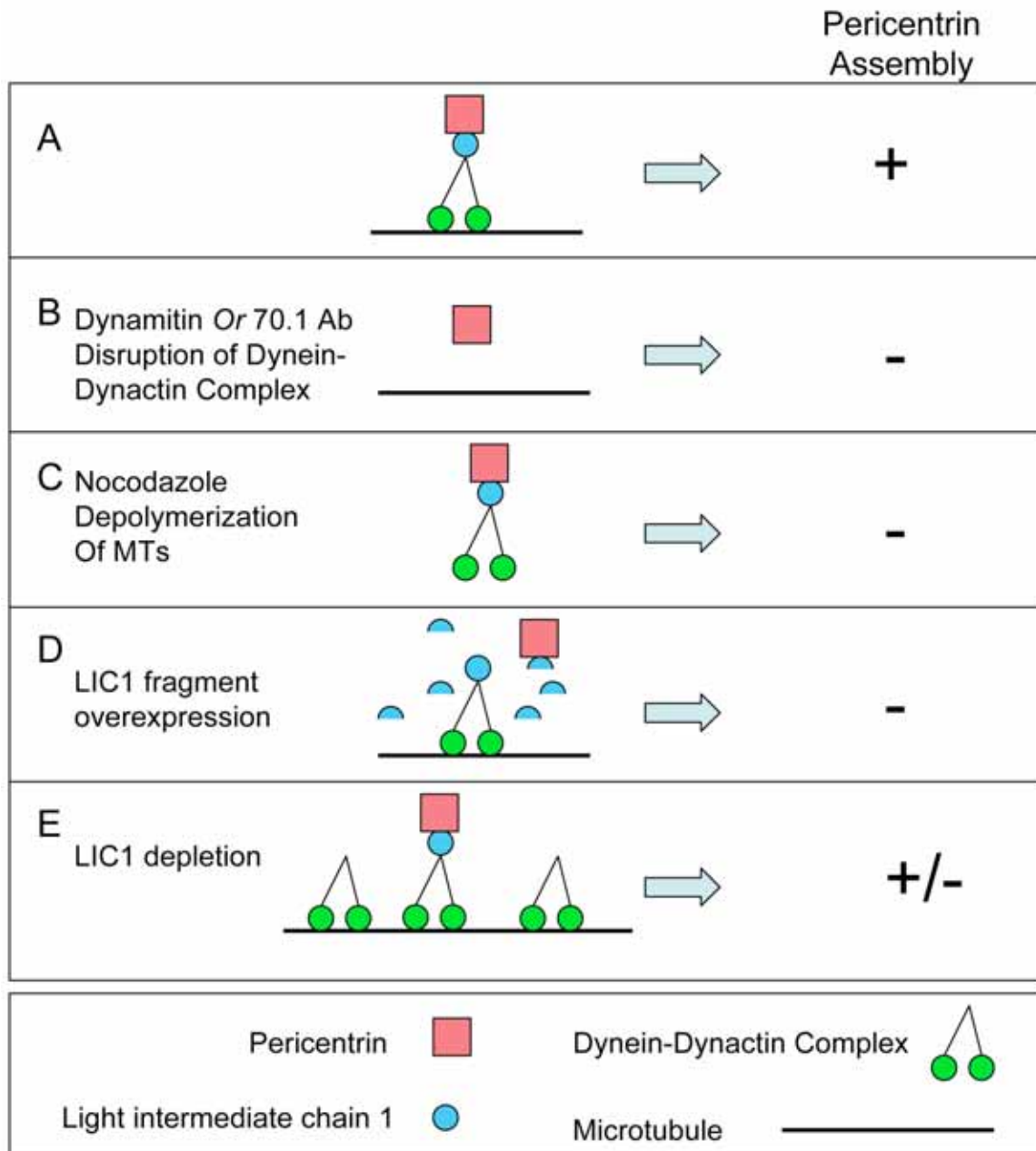


Figure 6: Pericentrin assembly model

A. LIC1 could act as a cargo adaptor for the dynein motor and allow pericentrin to bind to the dynein complex for assembly. **B.+C.** Pericentrin assembly to the centrosome has previously been shown to require dynein as well as microtubules (Young et al., 2000). **D.** The c-terminus of rLIC1 can bind to pericentrin but not to dynein, thus breaking up this

interaction and affecting pericentrin assembly. **E.** When LIC1 is depleted using RNAi, pericentrin assembles to a certain extent, as some LIC1 remains.

Discussion

Involvement of LIC1 in pericentrin assembly

The overexpression data for the c-terminus of rLIC1 shows that when this protein is overexpressed, pericentrin is unable to assemble to the centrosome. It does not appear that this assembly defect is tied to any problems with the microtubule cytoskeleton or the functionality of the dynein motor complex as these parameters are normal in cells overexpressing the c-terminus of rLIC1. Instead it appears that pericentrin is bound by the c-terminus of rLIC1, which does not associate with the dynein complex, efficiently keeping pericentrin away from dynein and inhibiting the assembly of pericentrin to the centrosome.

Depletion of LIC1 protein levels does lower levels of pericentrin at the centrosome, but not as much as one might expect with the 70-86% depletion of LIC1 protein observed. This can be explained in a few different ways. First, the LIC1 depletion is a depletion and not a knockout so there is still some LIC1 protein around and pericentrin may assemble well enough with this low level of LIC1. Second, as hLIC1 and hLIC2 are very similar in amino acid sequence it could be that LIC2 is able to step in when there is very little LIC1 and allow for the assembly of pericentrin to the centrosome. Pericentrin would be able to assemble with LIC1 depletion, but with the overexpression of the c-terminus of rLIC1, pericentrin would be sequestered away from endogenous LIC1-dynein as well as LIC2-dynein. This seems unlikely considering the published co-overexpression immunoprecipitation data showing pericentrin interacts with LIC1 and not LIC2 (Tynan

et al., 2000b). Third, pericentrin assembly may rely on several binding sites to different subunits of dynein. Pericentrin may be able to bind dynein fairly well in the absence of LIC1, but when bound to the c-terminus of rLIC1, pericentrin is structurally inhibited from interacting with dynein. Along with this idea of a second binding site on dynein it may not be pericentrin that has a second binding site for this other subunit of dynein. As pericentrin and gamma-tubulin appear to assemble as a complex to the centrosome (Dictenberg et al., 1998), it could be another member of the pericentrin and gamma-tubulin complex that has a binding site for dynein. In the absence of another known binding site for pericentrin or gamma-tubulin to dynein and no precedent for complexes binding multiple subunits of dynein, the simplest explanation for the depletion of LIC1 not having a strong effect on pericentrin assembly is that enough LIC1 is left to allow for fairly normal assembly.

In summary microtubules and cytoplasmic-dynein have been shown to be important for the assembly of pericentrin onto the centrosome (Young et al 2000). Pericentrin has been shown to interact with dynein and light intermediate chain 1 (LIC1) (Aruna et al, 1999; Tynan et al, 2000), suggesting a molecular means by which pericentrin would rely on dynein for assembly. Our hypothesis is that by overexpressing a dominant negative fragment of LIC1 that binds pericentrin but not dynein, pericentrin was unable to bind to a functional LIC1-dynein complex and unable to assemble onto the centrosome (see figure 6 for a model).

LIC1 as a cargo binder for dynein

This study expands the studied functions for LIC1. Previously LIC1 has been shown to be important for melanosome movements toward the centrosome (Reilein et al., 2003). The exact protein interaction involved in this process is unknown. The other proteins that LIC1 interacts with besides the heavy chain of dynein are Rab4A, a protein involved in regulating the endosome recycling pathway (Bielli et al., 2001), and pericentrin (Purohit et al., 1999). Our study shows that one function of the LIC1-pericentrin interaction is to assemble pericentrin to the centrosome. This is an important function as pericentrin is an important scaffold protein at the centrosome for a number of different functions. This is the first time that LIC1 has been demonstrated to be a protein cargo binder for dynein, rather than just being involved in a regulatory capacity for binding vesicles. The LICs binding protein cargoes for dynein is not unprecedented as dynein 2 LIC is involved in the dynein related movement of intraflagellar transport (IFT) proteins in flagella (Hou et al., 2004). In summary, our study has added pericentrin assembly to the growing list of functions for dynein 1 LIC1.

Materials and Methods

Cell Culture

COS (ATCC CRL-1651) and HeLa cells (ATCC CCL-2) were cultured as described by American Type Cell Collection. HeLa cells were only used in figures 4B and 5.

Constructs and overexpression

C-terminal myc tagged c-terminus of rLIC1, amino acids 174-523 (Tynan et al., 2000b) was used for this study. A beta-galactosidase vector was used as a control overexpression construct in figure 2 (Purohit et al., 1999). HA (Purohit et al., 1999) and Flag tagged pericentrin constructs were used for this study. Flag tagged pericentrin was generated by cloning full length pericentrin out of the HA-pericentrin vector into the pDNR-2 (Clontech Laboratories, Inc) and sequenced. A 3x flag sequence, several other tags and an inframe cre-lox site was inserted into the pEF6/V5-His B vector (Invitrogen). The cre-lox site allowed for the transfer of pericentrin from the pDNR-2 vector to the Flag vector according to the manual specifications for the creator system (Clontech Laboratories, Inc). Constructs were delivered to cells using Lipofectamine 2000 for the sucrose gradient data or Lipofectamin Plus (Invitrogen) for the co-overexpression immunoprecipitation data, both following the instructions in the accompanying manuals. Cytoplasmic microinjection of cDNAs were done as previously described (Young et al., 2000). Cells for the pericentrin assembly experiment in figure 2 were treated with thymidine for 16 hours to synchronize them in S phase. They were then released for 7 hours to move them into G2, about one hour shy of entering mitosis. Cells were then microinjected. Thymidine was added back two hours after microinjection and cells were

incubated with thymidine for 20 hours allowing for full pericentrin assembly. Cells for figures 3A and 4C and E were blocked in thymidine for 16 hours, released for one hour before microinjection and then were fixed 7 hours later.

siRNA treatment

siRNAs (21-nt; Dharmacon Research, Inc.) targeting hLIC1 (GenBank/EMBL/DDBJ accession no. NM_016141;nt 1156-1174), GFP (Gromley et al., 2003) were delivered to cells using Dharmafect 1 (Dharmacon Research, Inc) according to the manual protocol. GFP siRNA was used as a control siRNA. hLIC1 siRNA target sequence is specific to hLIC1 and does not completely match hLIC2.

In figure 5B the assembly assay for knockdown cells was done using a mitotic shake off. HeLa cells were transfected with control or LIC1 specific siRNAs. About twenty-six hours after transfection a preshake off was performed to remove any interphase cells that were not attached well. Two hours after this mitotic cells were taken with a shake off and plated on fibronectin (Calbiochem) coated coverslips. Cells generally have divided and adhered to the coverslips by two hours after plating, so cells were fixed and stained for pericentrin and 5051 so that early G1 centrosomal levels of pericentrin could be measured. Several coverslips were allowed to grow for 17 to 19 hours and then fixed and stained for pericentrin and 5051 so that G2 centrosomal levels of pericentrin could be measured. Net pericentrin assembly was measured by subtracting the early G1 levels from the G2 levels of pericentrin.

Antibodies, Fixation, Staining and Imaging

Cells were fixed in methanol (Dictenberg et al., 1998). The following antibodies were used: Anti dynein intermediate chain 74.1 and anti α -tubulin DM1 α culture supernatant (Sigma); anti myc 9E10 (Santa Cruz); 5051 serum (Tuffanelli et al., 1983). Golgi was directly stained with *Helix pomatia* agglutinin Alexa 488 (Invitrogen). Images were taken on a wide field microscope as previously described (Dictenberg et al., 1998). The entire fixed cell volume was imaged and displayed as a two-dimensional projection (MetaMorph; Universal Imaging Corp.). Pericentrin levels were integrated as previously described (Young et al., 2000). Net pericentrin levels were calculated by subtracting normal early G1 levels of pericentrin that are the lowest pericentrin levels normally seen in cells from other pericentrin levels to get the net increase in pericentrin.

Western Blotting

Proteins denatured in 2x SDS buffer (25% glycerol, 2% SDS, 0.01% bromophenol blue, 62.5mM Tris, pH6.8) were separated by SDS-PAGE, transferred to PVDF membranes and probed with the following antibodies: Anti dynein intermediate chain 74.1 and anti actin AC40 (Sigma); anti myc 9E10 (Santa Cruz); anti HA 12 CA5 (Covance); anti LIC1 (described in chapter 2). Anti-rabbit and anti-mouse HRP antibodies were used along with ECL plus luminescent reagent (Amersham Biosciences) and then visualized on film (Kodak).

Immunoprecipitation

Lipofectamine 2000 (Invitrogen) was used to transfect cells following the manual protocol. 24 hours after transfection, cells were lysed in 50mM Tris HCl pH 8.0, 150mM

NaCl, 1mM EGTA, and 1% IGEPAL CA-630. For every 10mls of lysis buffer, one complete protease inhibitor tablet (Roche) was added to inhibit a range of proteases. Immunoprecipitations were performed with anti myc 9E10 (Santa Cruz), anti HA 12CA5 (Covance), anti flag M2 (Sigma) antibodies and Protein G Plus-Agarose beads (Santa Cruz Biotechnology) overnight at 4°C with rocking of the tubes. Beads were then washed with lysis buffer and 2xSDS buffer was added to denature the proteins and allow for loading on an SDS-PAGE gel.

Sucrose gradient

Cells were harvested with 10mM EDTA in PBS. Cells were lysed in 50mM Tris-Cl pH 7.5, 150mM NaCl, 1% IGEPAL CA-630, 1mM EDTA, 1 complete protease inhibitor cocktail mini tablet (Roche) for each 10ml of lysis buffer. Lysates were spun at 16,000 RCF at 4°C for 10 minutes to pellet cellular debris. The supernatant was layered on a 12ml 5-20% sucrose gradient, and centrifuged at 4°C in a Beckman Coulter Optima L-90K ultracentrifuge (35,000 rpm, SW41 rotor) for 12.5 hours. Sedimentation standards used were thyroglobulin (19.4S), catalase (11.3S), and BSA (4.4S). Sucrose gradient fractions (500ul) were collected and analyzed by western blots.

Chapter 4: Light Intermediate

Chain 1 Insights Into Dynein

Function and Regulation

Spindle assembly checkpoint

For tens of years it has been known that drugs that affect the mitotic spindle will arrest cells in mitosis (Jordan, 1999; Levan, 1938). About twelve years ago an idea started to emerge that microtubule attachments to kinetochores was a key factor in this checkpoint (Rieder et al., 1994). This was soon followed by the identification of proteins, such as Mad1-Mad2 and BubR1, that are involved with producing a “wait anaphase” signal when localized to kinetochores that are not attached or under tension (Chan et al., 1999; Chen et al., 1996). There is a debate about the importance of Mad1-Mad2 versus BubR1 in this checkpoint (Hoyt, 2001; Shah and Cleveland, 2000; Shannon et al., 2002; Skoufias et al., 2001). It seemed that they are both needed at the kinetochore to efficiently produce the wait anaphase signal for two reasons. First, they both appear in the mitotic checkpoint complex that can inhibit the APC/C (Sudakin et al., 2001). Second, without either one of these proteins cells will go through mitosis into anaphase even after insults that normally delay cells via the spindle assembly checkpoint (Meraldi et al., 2004). One confounding issue in trying to separate the functions of Mad1-Mad2 and BubR1 is that in delayed cells

they are both present at kinetochores to some degree. Mad1-Mad2 is typically removed early, after microtubules are attached to kinetochores, whereas BubR1 is removed later, after sister kinetochores are under tension (Shannon et al., 2002; Skoufias et al., 2001; Taylor et al., 2001). Even with taxol checkpoint arrested cells, which appears to be predominantly a tension mediated checkpoint there are still a few kinetochores with Mad1-Mad2 (Waters et al., 1998). As these points illustrate, the importance of Mad1-Mad2 versus BubR1 in the spindle assembly checkpoint is hard to pin down. The results that are presented in chapter two show that kinetochore Mad1-Mad2 without BubR1 is sufficient to activate or maintain the spindle assembly checkpoint. BubR1 is still important to this checkpoint as without it cells go through the LIC1-dependent delay.

Dynein has also been shown to be involved in the spindle assembly checkpoint by moving checkpoint proteins, such as Mad1-Mad2 and BubR1, off kinetochores (Howell et al., 2000; Howell et al., 2001). The role of dynein in moving Mad1-Mad2 off of kinetochores is strengthened by the involvement of light intermediate chain 1 in this process. This data also adds detail to the movement of Mad1-Mad2 off kinetochores. This movement of Mad1-Mad2 not only requires LIC1, a specific subunit of dynein, but also requires the phosphorylation of LIC1 at a putative Cdk1 site. Further study of proteins that interact with the phosphorylated form of LIC1 and not with the unphosphorylated form will enhance our understanding of the proteins involved in the removal of Mad1-Mad2 from the kinetochore as well as how LIC1 facilitates the turning off of the wait anaphase signal.

It is interesting to note that LIC1 depletion does not interfere with BubR1 movement off kinetochores. This leaves open the possibility that another subunit of dynein is involved in the movement of BubR1 off kinetochores. The identification of this subunit would further our understanding of BubR1 removal from kinetochores and may give important information about how BubR1 senses tension.

Mitosis

Moving away from the spindle assembly checkpoint to mitosis in general, Cdk1 is the major mitotic kinase and has two general functions that appear to conflict with one another but really allow for a smooth transition from metaphase to anaphase once chromosomes are aligned at the metaphase plate. First, Cdk1 helps to set up and maintain the conditions of metaphase, such as chromosome condensation, assembly of the mitotic spindle, and strengthening of the spindle assembly checkpoint. Cdk1 strengthens the spindle assembly checkpoint by phosphorylating Cdc20 and weakening the interaction between Cdc20 and the APC/C (D'Angiolella et al., 2003). This allows unattached kinetochores to catalyze the Mad2-Cdc20 interaction, sequestering Cdc20 away from the APC/C preventing anaphase onset or the end of metaphase. Second, Cdk1 also sets up the mechanism by which the checkpoint is turned off. The putative phosphorylation of LIC1 by Cdk1 enables the movement of Mad1-Mad2 off kinetochores, as shown in chapter 2. The relocation of Mad1-Mad2 away from kinetochores disrupts the ability of free Mad2 to interact with or sequester Cdc20. When all kinetochores are attached to microtubules and dynein has moved Mad1-Mad2 off of all these kinetochores, then free Mad2 can no longer sequester Cdc20 from the APC/C and anaphase can be initiated.

The phosphorylation at S207 of rLIC1 is needed for the function of LIC1 in the spindle assembly checkpoint; however there are other phosphorylations to LIC1 in mitosis. There are three other suggested cdk1 phosphorylation sites (S398, S405 and T408) that could account for the entire shift that is seen for the LIC1 protein in mitosis (Addinall et al., 2001). These other phosphorylation sites could be necessary for the function of LIC1 in the spindle assembly checkpoint but they could also regulate other cargoes in mitosis. Another possible function for LIC1 in mitosis is spindle positioning. LIC1 does localize along the astral microtubules and it could be involved with spindle positioning although I did not observe any drastic problems with spindle position with the LIC1 depletion described in chapter 2. Regardless, the additional phosphorylations of LIC1 could be a key to the discovery of other functions for LIC1 in mitosis.

Pericentrin assembly

Pericentrin has been shown to assemble at the centrosome from early G1 until mitosis and then rapidly disassemble after mitosis to return to low levels of pericentrin at the centrosome to start the cycle again (Dictenberg et al., 1998). This assembly process has been shown to be dynein dependent (Young et al., 2000). The results discussed in chapter three support the idea that dynein is involved in the process of pericentrin assembly and move forward the idea that LIC1 is the particular subunit involved in targeting pericentrin as a cargo for dynein.

As the pericentrin binding domain contains the S207 phosphorylation site it is possible that pericentrin binding is regulated in some way by phosphorylation of this site. It appears that the majority of pericentrin assembly occurs before entry into mitosis (Dictenberg et al., 1998), so it is possible that pericentrin binds the unphosphorylated form of LIC1. This would allow pericentrin to assemble in interphase and then free LIC1 for other functions in mitosis such as its involvement in the spindle assembly checkpoint. Another possibility is that phosphorylation of S207 does not affect pericentrin binding to LIC1 and that there are other ways to regulate pericentrin binding to LIC1. The last possibility is that LIC1 can be phosphorylated at the S207 site in interphase as well as mitosis. There are two reports that suggest the presence of this phosphorylation in interphase. First, this site on endogenous *Xenopus* LIC is shown to be phosphorylated in *Xenopus* extracts by mass spectrometry (Addinall et al., 2001). Second, in interphase neuron preps there is a shifted form of LIC1 and LIC2 that could be correlated with the shift seen for the S207 site (Dillman et al., 1996). As Cdk1 is only really active in mitosis (Kramer et al., 2004), another kinase would have to phosphorylate this site in interphase, such as MCAK. If this site were phosphorylated in interphase and pericentrin bound to the phosphorylated form, it could explain pericentrin assembling at different rates during the different phases of the cell cycle (Dictenberg et al., 1998) as more LIC1 could be phosphorylated during the different cell cycle phases. This is an interesting possibility as it integrates the phosphorylation site and the demonstrated pericentrin interaction domain on LIC1.

Interphase

The phosphorylation of the rLIC1 S207 site in interphase could potentially regulate other interactions as well. LIC1 has been shown to interact with Rab4A, which is involved in the recycling endosome pathway (Bielli et al., 2001). The Rab4A binding site on LIC1 overlaps with the pericentrin binding site at the S207 phosphorylation site. These two cargoes appear from every indication to be interphase cargoes, so it is possible that LIC1 would only be able to bind one of these at a time and that the interphase phosphorylation of this site may be involved in regulating the binding of one of these cargoes over the other. For example it could be that LIC1 would preferentially bind Rab4A when unphosphorylated at this site and pericentrin when phosphorylated at this site. Then when cells do enter mitosis the majority of LIC1 is phosphorylated at S207 by Cdk1 and so Rab4A, a vesicle regulatory protein, would be lost from LIC1 in co-ordination with dynein being lost from membrane fractions (Addinall et al., 2001). Further work on the S207 site in rLIC1 and interactions with Rab4A and pericentrin could demonstrate the regulation of cargoes to one subunit of dynein.

Dynein

In chapter one different phosphorylations of dynein were divided into three basic categories depending on the effect of the phosphorylation. First, a phosphorylation event can result in the gain or loss of dynein subunits therefore affecting what cargoes can interact with the motor domain of dynein. Second, a phosphorylation event can result in the gain or loss of the ability of dynein to move along microtubules. In this case dynein

does not disengage from its cargo. Third, a phosphorylation event can result in the direct change of a cargo binding site resulting in a shift in the cargoes targeted by that particular dynein subunit. The conclusion in chapter two that the direct or indirect Mad1-Mad2 interaction with dynein involves the phosphorylation of LIC1 strengthens the idea that phosphorylation events on dynein can regulate cargo binding directly.

The LIC1 subunit of dynein is set up to have multiple cargoes with regulation of these interactions through phosphorylation and this has implications for the interactions and regulation of LIC2. As previously discussed in this chapter LIC1 appears to interact with pericentrin, Rab4A and Mad1-Mad2. The association of LIC1 with Mad1-Mad2 can be regulated with phosphorylation of the rLIC1 S207 site and the pericentrin and Rab4A interaction domains on LIC1 overlap on this same phosphorylation site. In chapter one it was also shown that an area of low homology between LIC1 and LIC2 overlapped this region where LIC1 appears to have regulation of multiple different cargoes. Again the phosphorylation site is conserved in LIC2 allowing for regulation, and the region around the phosphorylation site is different than LIC1 allowing for many as yet undiscovered cargoes and functions for LIC2. LIC2 forms a separate class of dynein that does not include LIC1 (Tynan et al., 2000b), so there is a separation of the cargoes of these two light intermediate chains to different dynein complexes. As LIC2 has unique localization within neurons this would be one place to look for unique cargoes, but this would only show the neuronal interphase cargoes for LIC2. As LIC2 is found in many different tissues (Hughes et al., 1995) and is set up like LIC1 to have mitotic specific cargoes,

there could be a range of cargoes that interact with LIC2 with a range of functions which would need to be regulated.

The study of the S207 rLIC1 phosphorylation site in chapter two provides a unique way to study dynein functions in mammalian cells. The functions of cytoplasmic dynein in mammalian cells have generally been studied by disconnecting dynactin with antibodies or by overexpressing dynamitin, a subunit of dynactin (Burkhardt et al., 1997; Karki and Holzbaur, 1995; Steffen et al., 1997; Vaughan and Vallee, 1995). Dynactin is a huge multi-protein complex involved in both the processivity of the motor as well as targeting of dynein to vesicles (King and Schroer, 2000; Steffen et al., 1997). The loss of dynactin represents the loss of the ability of dynein to efficiently move cargo along microtubules as well as to attach to many vesicle cargoes. While disruption of the dynein-dynactin interaction may be a good way to look for dynein phenotypes in general, it cannot be used to find the specific subunit involved in a particular function. The depletion of LIC1 did not alter the function of dynein in general so the further investigation of functions to this particular subunit was possible. It is probable that the light chains are also not structurally or biochemically needed for general dynein functionality so it would be possible to study functions particular to these subunits in the same way. Also further investigation of particular sites, such as phosphorylation sites or cargo interaction sites, is possible by overexpressing point mutants that disrupt these regions while depleting the endogenous subunit, as was done to look at the involvement of the S207 phosphorylation site on LIC1.

Concluding remarks

To conclude, LIC1 is shaping up as a multifunctional cargo binder for cytoplasmic dynein 1. LIC1 is shown here to be involved in pericentrin assembly, and in turning off the wait anaphase signal of the spindle assembly checkpoint. These functions are new additions to the list of LIC1 functions. Further study of LIC1 will help with the understanding of how LIC1 is regulated to target different cargoes in interphase and mitosis as well as within each of these cell cycle stages. Study of LIC2 will undoubtedly provide some interesting new cargoes and functions for dynein that are unique from LIC1 but regulated in a similar fashion as it appears that the regulatory features have been conserved between LIC1 and LIC2. The study of the light intermediate chains of cytoplasmic dynein 1 will bring to light new functions that dynein is involved in that might not be obvious when using blunt tools that disrupt all cytoplasmic dynein 1 functions within the cell. A greater understanding of the functions and interactions of LIC1 versus LIC2 within the same cell will also allow for a greater understanding of how dynein can co-ordinate or regulate so many different functions.

Abbreviations list

APC/C – Anaphase promoting complex/cyclosome

AZ/DOG – Azide, 2-deoxyglucose and oxyrase

Cdk1 – Cyclin dependent kinase 1, cdc2-Cyclin B1

DYNC1H1 – Cytoplasmic dynein 1 heavy chain

DYNC1I1 – Cytoplasmic dynein 1 intermediate chain 1

DYNC1I2 – Cytoplasmic dynein 1 intermediate chain 2

DYNC1LI1 – Cytoplasmic dynein 1 light intermediate chain 1

DYNC1LI2 – Cytoplasmic dynein 1 light intermediate chain 2

DYNLL1 – Dynein LC8 light chain 1

DYNLL2 – Dynein LC8 light chain 2

DYNLRB1 – Dynein Roadblock light chain 1, Robl1

DYNLRB2 – Dynein Roadblock light chain 2, Robl2

DYNLT1 – Dynein Tctex1 light chain 1, Tctex-1

DYNLT3 – Dynein Tctex1 light chain 3, rp3

IB – Immunoblot

IC-1 – Cytoplasmic dynein 1 intermediate chain 1 (DYNC1I1)

LIC1 – Cytoplasmic dynein 1 light intermediate chain 1 (DYNC1LI1)

LIC2 – Cytoplasmic dynein 1 light intermediate chain 2 (DYNC1LI2)

NEB – Nuclear envelope breakdown

TGFb – Transforming growth factor beta

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