THE DYNLT3 LIGHT CHAIN DIRECTLY LINKS CYTOPLASMIC DYNEIN TO A SPINDLE CHECKPOINT PROTEIN, BUB3*

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Running title: Binding of the dynein DYNLT3 light chain to Bub3

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Cytoplasmic dynein is the motor protein responsible for the intracellular transport of various organelles and other cargoes towards microtubule minus-ends. However, it remains to be determined how dynein is regulated to accomplish its varied roles. The dynein complex contains six subunits, including three classes of light chains. The two isoforms of the DYNLT (Tctex1) family of light chains, DYNLT1 and DYNLT3, have been proposed to link dynein to specific cargoes. However, no specific binding partner had been found for the DYNLT3 light chain. We find that DYNLT3 binds to Bub3, a spindle checkpoint protein. Bub3 binds exclusively to DYNLT3, and not to the other dynein light chains. GST pull-down and co-immunoprecipitation assays demonstrate that Bub3 interacts with the cytoplasmic dynein complex. DYNLT3 is present on kinetochores at prometaphase, but not later mitotic stages, demonstrating that this dynein light chain, like Bub3 and other checkpoint proteins, is depleted from the kinetochore during chromosome alignment. Knockdown of DYNLT3 with siRNA increases the mitotic index, in particular, the number of cells in prophase/prometaphase. These results demonstrate that dynein binds directly to a component of the spindle checkpoint complex through the DYNLT3 light chain. Thus, DYNLT3 contributes to dynein cargo binding specificity. These data also suggest that the subpopulation of dynein, containing the DYNLT3 light chain, may be important for chromosome congression, in addition to having a role in the transport of checkpoint proteins from the kinetochore to the spindle pole.

Cytoplasmic dynein 1 is a large multi-subunit ATPase which moves toward the minus ends of microtubules. It is involved in multiple important cellular processes including mitosis, nuclear migration, Golgi and centrosome localization, organelle transport, and viral transport (1,2). This dynein complex has a molecular mass greater than 1 MDa and contains two identical heavy chains, each containing motor domains that generate the force necessary to move along the microtubule (3-5). The cytoplasmic dynein cargo binding domain consists of five subunits that assemble into the complex as dimers: the intermediate chain, light intermediate chain, and three classes of light chains; DYNLL, DYNLT, and DYNLRB1. There are at least two isoforms of each of these five subunits (6). The DYNLT light chain family has two members, DYNLT1 (previously called Tctex1) and DYNLT3 (previously called rp3) (2,7-9). The two family members share ~55% amino acid identity. Both light chains are expressed in all the cells and tissues so far examined, and they incorporate into dynein complexes as homodimers [(7,10) and our unpublished observations]. Thus, a dynein complex contains either DYNLT1 or DYNLT3, but not both.
While the dynactin complex is responsible for binding dynein to many cargoes, there is increasing evidence that the isoforms of cargo binding subunits are important in binding dynein to specific cargoes [reviewed in (1)]. DYNLT1 has been shown to specifically interact with a number of proteins. For example, DYNLT1 binds to rhodopsin and Doc2 whereas DYNLT3 does not (1,11,12). The discovery of a large number of functionally unrelated proteins that bind to DYNLT1 led to the suggestion that the DYNLT light chains function as adaptors to link specific cargoes to the dynein motor (1,6,13-15). While this hypothesis is supported by the report that both DYNLT3 and DYNLT1 bind to the HSV-1 capsid protein (16), to date no specific cargo for the DYNLT3 light chain has been identified. To gain insight into the cargo binding function of the DYNLT3 light chain, we performed a yeast two-hybrid screen to identify novel DYNLT3 binding proteins. One of the putative DYNLT3 interacting proteins was Bub3, a component of the spindle checkpoint complex (17). The Bub3 gene (budding uninhibited by benzinidazoles 3) was first identified in yeast (18).

The spindle checkpoint is a signal transduction pathway that blocks mitosis until all kinetochores are attached to spindle microtubules [reviewed in (19-22)]. Bub3 is a conserved essential component of the checkpoint and it is a scaffold that promotes interactions between the other spindle checkpoint proteins, including Bub1, Mad2, Cdc20, BubR1/Mad3 (17,23-26). Recently, RNAi has been used to reduce the level of spindle checkpoint proteins in cells. These experiments have shown that some of the proteins have important roles early in mitosis, prior to their involvement in the checkpoint complex. For example, when Bub1 is depleted, cells are more likely to have misaligned chromosomes and this defect has been linked to Bub1's role in maintaining centromeric cohesion (33,34). Also, RNAi reduction of Bub3 levels leads to the accumulation of cells in prophase (24). Cytoplasmic dynein is thought to contribute to the inactivation of the spindle checkpoint by transporting checkpoint proteins from kinetochores to the spindle poles along the newly attached kinetochore microtubules (27-32). However, the molecular basis underlying the association of checkpoint proteins with dynein was unknown.

We find that the cytoplasmic dynein light chain DYNLT3 binds to Bub3 in vivo and in vitro and that Bub3 does not bind to the related light chain DYNLT1 or any other dynein light chains. We further show that GST-Bub3 binds to the dynein complex, and that Bub3 and Mad2 co-immunoprecipitate with dynein from mitotic cell extracts. DYNLT3 co-localizes with Bub3 at kinetochores in prometaphase, but not at later mitotic stages. siRNA knockdown of the DYNLT3 light chain results in a 3 fold increase in the mitotic index, and an increase in the number of cells in prophase/prometaphase. Our data thus strongly support the hypothesis that the two DYNLT light chain family members contribute to the differential regulation of dynein cargo binding, and that the interaction of Bub3 and DYNLT3 links a checkpoint protein complex to a subpopulation of dynein.

**EXPERIMENTAL PROCEDURES**

**Plasmids** - pBluescript-SK-DYNLT3 encoding mouse DYNLT3 (Open Biosystem) served as a template for the PCR amplification. To minimize autoactivation by DYNLT3 in yeast two-hybrid assays [our observation and (16)], a flexible linker "--Ser-Gly-Gly-Ser-Gly-Gly--" was inserted in front of the ORF of DYNLT3 by polymerase chain reaction, PCR, and the product was cloned into the BamHI/Xhol sites of the pGBKTK7 vector (Clontech). For expression of the rat DYNLT1, DYNLT3, DYNLL1 (previously called LC8a), DYNLL2 (previously called LC8b), DYNLRB1 (previously called Roadblock1), and DYNLRB2 (previously called Roadblock2) light chains in cultured mammalian cells, their entire coding regions were individually cloned into the pCMV-myc vector (Clontech). Human DYNLT3-GFP was made by subcloning the DNA fragment into pEGFP vector (Clontech). A 6-His N-terminal tag
was added to human DYNLT3 by cloning it into pET-14b (Novagen Inc.). The entire coding region for human Bub3 was PCR-amplified using Bub3-p3xFLAG-CMV (the gift of Dr. Tim Yen, Fox Chase Cancer Center, Philadelphia, PA) as a template. The PCR product was cloned into EcoRI and SalI sites of glutathione-S-transferase (GST)² pGEX4T-3 vector (Amersham Biosciences). Mouse Bub3 N-terminal fragment (aa 1-166) isolated from the yeast two-hybrid screen was excised from NotI sites of the Gal4 activation domain vector, pVP16, and subcloned into the GST fusion vector, pGEX4T-1 (Amersham Biosciences), GST-Bub3NT. All constructs were sequenced to confirm gene sequence and correct reading frame. 

**Yeast two-hybrid screen** - DYNLT3 in pGBKT7 vector was used to screen screen ~1 × 10⁶ clones from a mouse embryonic cDNA library constructed in pVP16 vector (35) (the gift of Dr. Ian Macara, University of Virginia). The yeast strain (AH109) used for the screening assay contained both HIS3 and lacZ reporter genes under control of a Gal4-responsive upstream activation site. Bait and library vectors were co-transformed into AH109 and positive interactions initially identified by growth of cells on media lacking leucine, tryptophan, and histidine (referred to as -His) with 5 mM 3-AT, an inhibitor of histidine synthase, at 30°C for 3 to 4 days. Positive colonies obtained from the screen were confirmed by β-galactosidase activity assay. To minimize the number of false positives and to increase the likelihood of obtaining pure clones, the initial isolates were grown in -His media with 30 mM 3-AT. Inserts from individual positive clones were isolated by direct PCR screening using flanking primers specific for the pVP16 plasmid. All of the PCR products were sequenced and potential DYNLT3-interacting clones were identified using NCBI Blast. The colony inhibition assay using 3-AT described in (36) was used to confirm the specificity of the putative interactions.

**Expression and purification of fusion proteins** - Bacterial expression of GST-recombinant proteins was carried out using *Escherichia coli* BL21(DE3) as host cells. To minimize the formation of inclusion bodies, protein expression continued for 10-12 h at 18°C before harvesting by centrifugation. After lysis, soluble proteins were purified by using a glutathione-Sepharose (GSH) affinity column (Amersham Biosciences) following the manufacturer's instructions. The purified GST fusion proteins were kept on beads in phosphate-buffered saline (PBS) including protease inhibitors (described below) and were directly used for binding assay experiments. GST fusion protein concentrations were estimated by comparing the intensity of the GST fusion protein to a series of different concentration BSA protein standards. Expression of the His-tagged DYNLT3 in bacteria was performed according to the manufacturer's instruction (Qiagen).

**GST pull-down experiments** - The GST pull-down assays between GST-Bub3 and overexpressed light chains were performed and described in (37,38). Briefly, at least ten micrograms of GST or GST fusion proteins prebound to GSH beads were incubated with 100-120 mg of the cell lysates in appropriate binding buffers. After incubation for 3 h at 4 °C, the pelleted beads were washed extensively with the binding buffers and subsequently boiled with SDS-PAGE sample buffer. The proteins were first resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently analyzed by immunoblot analysis. Buffer A (PBS pH 7.4, 0.1% Triton X-100, 1 mM EDTA, 1 mM phenylmethysulfonyl fluoride, 1 mM benzamidine, and 1 μg/ml each leupeptin and pepstatin) was used to characterize the interaction between GST fusion proteins and the overexpressed light chains. Buffer B (50 mM PIPES, 50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 3 μg/ml each leupeptin and pepstatin) was used to characterize the interaction between GST fusion proteins and endogenous dynein subunits. For the direct in vitro binding assay, 10 μg of GST or GST-tagged proteins prebound to GSH beads were incubated with 1 ml of bacterial lysates containing His-DYNLT3 in
the buffer A. After incubation at 4 °C for overnight, the beads were extensively washed with the buffer A, and the bound proteins were subsequently released by boiling with SDS-PAGE sample buffer. The released proteins were resolved by SDS-PAGE and detected on Western blots.

**Polyacrylamide gel electrophoresis, and immunoblotting** - SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (37,38).

**Cell culture and transfection** - COS7 (monkey kidney) and NRK (normal rat kidney) cells were grown in Dulbecco’s modified essential medium (DMEM) containing 10% calf serum (HyClone). LLCPK (porcine kidney epithelial) cells were cultured in F-12 medium containing 10% fetal bovine serum. For transient transfection, cells were transfected with the Fugene 6 transfection kit (Roche Molecular Biochemicals) or the nucleofector (Amaxa Biosystems), following the manufacturer’s instructions. Transfected cells were allowed to grow for 24 to 36 h. Transfections were performed at 80% confluence.

**Immunoprecipitation** - A mitotic NRK cell lysate was prepared by the method of Nakagawa (39). Briefly, cells were incubated with 2 mM thymidine for 10 to 12 h to arrest the cells in S phase. The cells were then arrested in mitosis by incubating with (1.7 μM) nocodazole for another 12 h. The mitotic cells were harvested by a “shake-off” procedure. The cells were washed and then lysed in Buffer B by repeated passages through a needle. The mitotic extract was recovered by ultracentrifugation of the lysed cells. To obtain an interphase extract, unsynchronized NRK cells were harvested by a “shake-off” procedure. The cells were washed and then lysed in Buffer B by repeated passages through a needle. The mitotic extract was recovered by ultracentrifugation of the lysed cells. To obtain an interphase extract, unsynchronized NRK cells were harvested by scraping. The lysates were clarified by centrifugation and then used for immunoprecipitation with antibodies, as indicated, and protein A-Sepharose beads (Zymed). After extensively washing the beads with Buffer B, the precipitated proteins were resolved by SDS-PAGE and detected by Western blotting with the indicated antibodies.

**siRNA** - DYNLT3 siRNA mixture containing (5’-GCAAGCAUCGUUGAAACAUAG-3’; 5’-GGCUUCAUGCUGAGGAAGCACAUA-3’; and 5’-GGCUAACGUCGAGGAACGUAAUA-3’) and control siRNA (5’-UAUGUGCUUCCUCAGCAUGAGCC-3’). siRNA transfections were carried out in 2 x 10^6 NRK cells using the nucleofector according to the manufacturer’s protocol. Briefly, the cells, in the solution V, were co-transfected with 3 μg of DYNLT3 siRNA or control siRNA and 0.5 μg of pEGFP-N1 (Clontech) to ensure that cells expressing GFP were co-transfected with siRNA. Cells were allowed to recover for 48 hours before subsequent manipulations.

**Antibodies and Immunocytochemistry** - The following primary mouse monoclonal antibodies were used: anti-Bub3 (BD Transduction Laboratory); R1, anti-DYNLT3 (Lo et al. in preparation); 9E10, anti-myc antibody; 12C5, anti-HA, (from the Lymphocyte Culture Center, University of Virginia, Charl. VA); IC74.1, anti-dynein intermediate chain (40); anti-His antibody (Upstate Biotechnology, Inc); anti-tubulin; Tu-27 for immunoblotting (41,42), and DM1A for immunocytochemistry (Sigma); also rabbit anti-dynein heavy chain antibody (gift of Dr. Richard Vallee, Columbia University); rabbit anti-Mad2 ([43), the gift of Dr. T. Salmon]; and human anti-Crest (Antibodies Incorporated). LLCPK cells were grown on coverslips, transfected with DYNLT3 fusion proteins and processed for immunocytochemistry (44). Briefly, the cells extracted with 1% TX-100 in PHEM for one minute, and immediately transferred to 4% paraformaldehyde in PHEM for 20 mins. Cells were then rinsed in PBS and blocked with normal goat serum and fish scale gelatin in Tris-buffered saline. Cells were incubated with primary antibody diluted in normal goat serum for 1 h. For secondary fluorescent staining, anti-rabbit, -human, and -mouse Cy3 or FITC antibodies (Jackson ImmunResearch Labs) were used. Coverslips were incubated with 4′,6-diamidino-2-
phenylindole (DAPI, 0.1 μg/ml) for 30 secs and mounted using Prolong antifade reagent (Molecular Probes). For prometaphase kinetochore staining, cells were also pre-incubated with 0.17 μM nocodazole for 20 min prior to processing. Images of cells were acquired with a Nikon Diaphot microscope equipped with a CoolSnapES CCD camera (Photometrics) controlled by MetaMorph software (Universal Imaging). MetaMorph and Adobe Photoshop 7.0 (Adobe Systems) were used to process all images.

RESULTS

The cytoplasmic dynein light chain, DYNL3T, interacts with the spindle checkpoint protein, Bub3. In preliminary experiments using DYNL3T in pairwise yeast two-hybrid assays it was found that DYNL3T fused to the DNA binding domain was a strong auto-activator of histidine synthase [our unpublished observations and (16)]. Therefore, in order to use a yeast two-hybrid library screen to identify DYNL3T binding partners, it was first necessary to engineer a construct that minimized the auto-activation. We found that adding a flexible linker “-Ser-Gly-Gly-Ser-Gly-Gly-” between the DNA binding domain and DYNL3T reduced the auto-activation to very low levels (data not shown). The screening of a mouse embryonic library resulted in the isolation of 99 independent clones. 51 clones from the primary isolate were identified as strong interactors with the β-galactosidase activity assay. Of these, 27 were able to grow in -His medium with high concentration of 3-AT (30 mM). Sequence analysis of these 27 clones indicated that 9 were membrane associated proteins, 6 were novel proteins and 11 were enzymes/structural proteins. DYNL3T was also identified in the screen consistent with the finding that it forms homodimers (10).

One candidate DYNL3T binding partner identified in the yeast two-hybrid library screen was the N-terminal portion of mouse Bub3 (aa 1-166). The strong specificity of the interaction of the two proteins was confirmed by using the full length human Bub3 in a pair wise yeast two-hybrid screen with DYNL3T (Figure 1A) in a colony inhibition assay (36). The specificity was further confirmed by assaying for the independent expression of β-galactosidase in the transformants (Figure 1B).

To demonstrate that the interaction between Bub3 and DYNL3T observed in the yeast two-hybrid assay was not mediated by a third yeast protein, the ability of bacterially expressed DYNL3T and Bub3 to interact was examined. GST-Bub3 was purified from a bacterial expression system and mixed in vitro with His-tagged DYNL3T also from a bacterial expression system. As can be seen in Figure 1C, the His-DYNL3T binds to and co-pellets with GST-Bub3 demonstrating that the purified proteins interact directly. We next sought to determine if Bub3 interacted with the other DYNLT family member, DYNL1T. DYNL1T and DYNL3T were tagged with the myc epitope and individually expressed in COS7 cells. The cells were lysed and incubated with GST-Bub3 bound to GSH beads in a pull-down assay. The data presented in Figure 2 show that Bub3 bound exclusively to DYNL3T. The same approach was used to show that Bub3 does not interact with any of the other dynein light chains, further demonstrating the specificity of the interaction between DYNL3T and Bub3 (Figure 2).

Bub3 and Mad2 bind to the cytoplasmic dynein complex. To demonstrate that the DYNL3T light chain is a link between Bub3 and the dynein complex, we employed two approaches. GST-Bub3 and GST were incubated with lysates from mitotic normal rat kidney (NRK) cells, and the bound proteins were analyzed by SDS-PAGE and Western blot (Figure 3A). As expected, DYNL3T from the mitotic extract bound to the Bub3 beads but not the GST beads. The dynein intermediate and heavy chains were also bound to the GST-Bub3 beads. The intermediate chain binds both DYNL3T and the heavy chain. Therefore, the presence of both the intermediate chain and heavy chain in the GST pull-downs is diagnostic for the presence of an intact dynein complex. This demonstrates that the dynein complex from mitotic cell extracts is capable of interacting with Bub3. To further
confirm interaction of dynein with Bub3, an antibody to the intermediate chain was used to immunoprecipitate the entire dynein complex (40,45). Bub3 from mitotic cell lysates was present in the dynein immunoprecipitate (Figure 3B) further supporting the conclusion that Bub3 interacts with the dynein complex. Bub3 also forms a complex with other checkpoint proteins, including Mad2. We also found that Mad2 co-immunoprecipitated with dynein from mitotic cell extracts (Figure 3B). To test whether the interaction of DYNLT3 and Bub3 was specific for mitotic cells, we analyzed dynein immunoprecipitated from unsynchronized, NRK cells, most of which are in interphase. The data in Figure 3B demonstrates that Bub3 was not found in the dynein immunoprecipitate.

**DYNLT3 co-localizes with Bub3 at prometaphase kinetochores.** The fact that Bub3 binds to DYNLT3 and not to DYNLT1 suggested that DYNLT3 serves as the link between a subpopulation of cytoplasmic dynein and a checkpoint protein complex. We therefore analyzed the distribution of DYNLT3-GFP in cultured cells to determine if its location was consistent with this role. During interphase, the DYNLT3-GFP fluorescence signal was distributed throughout the cytoplasm similar to the cytoplasmic distribution observed when cells are transfected with a GFP-tagged dynein intermediate chain [not shown, and see (46)]. During prometaphase, DYNLT3 was observed at kinetochores and co-localized with Bub3 (Figure 4A). We were unable to identify any DYNLT3 light chain associated with kinetochores of metaphase, anaphase or telophase cells (Figure 4B). Rather, DYNLT3 was found along astral and spindle microtubules (Figure 4C, D). Identical results were obtained regardless of the cell line used (NRK) or the use of myc and HA epitope tags (data not shown). We were also unable to detect Bub3 on kinetochores after prometaphase (Figure 4D). These observations indicate that the dynein variant with the DYNLT3 light chain associates with kinetochores during prometaphase but not during other phases of the cell cycle, and they are consistent with the hypothesis that the subpopulation of dynein with DYNLT3 is moving off of the kinetochore prior to metaphase.

**Knockdown of endogenous DYNLT3 with siRNA slows progress through mitosis.** To determine if the dynein variant with the DYNLT3 light chain has a role in mitosis consistent with disruption of checkpoint protein function, we used siRNA to reduce the expression of this light chain in NRK cells. We confirmed with RT-PCR that both DYNLT1 and DYNLT3 are expressed in this cell line (not shown). The efficient depletion of DYNLT3 protein by treatment of cells with siRNA oligonucleotides, compared to the control scrambled oligonucleotide, was demonstrated by Western immuno-blot analysis (Figure 5A). DYNLT1 remains in the cells after DYNLT3 siRNA (data not shown). Therefore, only DYNLT3 is depleted by this procedure. We then compared the relative number of mitotic cells in siRNA treated and control cells. SiRNA knockdown of DYNLT3 resulted in a 3 fold increase in the mitotic index (Figure 5B, p<0.002, Students t-test) and an almost 5 fold increase in the number of prophase/prometaphase cells relative to controls (Figure 5C, p<0.0002, Student's t-test). Interestingly, we observed no significant increase in the number of abnormal spindles in siRNA compared to control cells. These results indicate that dynein with DYNLT3 is necessary for efficient progression through mitosis, and suggest that it has a role in an early stage of mitosis.

**DISCUSSION**

It has been proposed that the two members of the DYNLT light chain family, DYNLT1 and DYNLT3, are important for binding dynein to specific cargoes (1,7,10). Supporting this model were two observations: that both light chains were expressed in all tissues and cultured cells examined, and that a wide range of proteins bound to DYNLT1 including rhodopsin (11), voltage gated calcium channel (47), Doc2β (12), heat shock protein PBP74 (48), and CD155, the polio virus receptor (49). The major weakness of the model was that no specific cargo for
DYNLT3 had been identified. In this study, we demonstrate that DYNLT3 links cytoplasmic dynein directly to Bub3, a spindle checkpoint protein. This conclusion is supported by a yeast two-hybrid pair wise interaction assay and an in vitro binding assay. The interaction of bacterially expressed Bub3 and DYNLT3 indicates that the binding of the two proteins is direct rather than through a third polypeptide. GST-Bub3 also bound to DYNLT3 over-expressed in cultured cells, not DYNLT1 or the other four dynein light chains. Furthermore, Bub3 and its binding partner Mad2 co-immunoprecipitated with cytoplasmic dynein from mitotic cell extracts, and dynein from mitotic cell extracts co-pelleted with GST-Bub3. Bub3 did not co-immunoprecipitate with dynein from interphase extracts. We also found that DYNLT3, like Bub3, is enriched on mammalian prometaphase kinetochores, but not metaphase or anaphase kinetochores. Depletion of DYNLT3 with siRNA increased the mitotic index, with an accumulation of cells in prophase/prometaphase. Our data demonstrates that dynein with DYNLT3 has functional importance for progression through mitosis. Our data also strongly support the model for dynein binding to different cargoes though the two DYNLT light chains.

The knockdown of DYNLT3 levels with siRNA suggests that the dynein subpopulation with DYNLT3 has a role in prophase/prometaphase, and possibly in chromosome congression. Interestingly, it has recently been observed that RNAi knockdown of Bub3 leads to an accumulation of cells in prophase (24). Misaligned chromosomes are also observed, suggesting that Bub3 may be involved in kinetochore-microtubule attachment (33,34). Kinetochore dynein is thought to be responsible for moving newly attached chromosomes along astral microtubules to the spindle poles (50). But the two sets of RNAi knockdown data suggest that dynein, together with Bub3, may also be involved in the formation of stable bipolar kinetochore attachments to microtubules and congression. An increase in the number of cells in metaphase was not observed after DYNLT3 depletion. This may be because the accumulation of cells in prometaphase masked putative effects on metaphase. GFP-Mad2 has been observed moving from kinetochores along microtubules to spindles poles in a dynein dependent manner (27). Our immunocytochemical data, and the finding that Bub3, Mad2 and dynein co-immunoprecipitate, may provide a molecular explanation for dynein-mediated checkpoint protein translocation from the kinetochore upon microtubule attachment. Bub3 and DYNLT3 could function as scaffolds directly linking dynein to the checkpoint protein complex for kinetochore to pole transport.

Cytoplasmic dynein participates in several other stages of the mitotic process. Dynein is important for spindle assembly (51). In Drosophila it is involved in chromosome to pole movement during anaphase A (52). Dynein in the cell cortex is positioned to orient the spindle and to pull the spindle poles apart in anaphase B (53-55). Our immunocytochemical and siRNA data suggest that different subpopulations of dynein may be responsible for some of these roles. The dynein variant with DYNLT3 does not appear to be involved in spindle assembly, as we observed no difference in the number of abnormal spindles after siRNA reduction of DYNLT3 levels. This is in contrast to the substantial increase in the number of abnormal spindles observed when dynein is inhibited by over-expression of either the p50 (dynamitin) subunit of dynactin or the CC1 fragment of the p150 subunit of dynactin (56-57). We were also unable to detect any DYNLT3 on kinetochores after prometaphase. This suggests that dynein with DYNLT3 is not on kinetochores and so would not participate in chromosome to pole movement.

In conclusion, we have shown that the cytoplasmic dynein light chain DYNLT3 and Bub3 co-localize on prometaphase kinetochores; that DYNLT3, binds to the checkpoint protein Bub3 in vitro; and that GST-pull-down and co-immunoprecipitation assays demonstrate the interaction between Bub3 and Mad2 with cytoplasmic dynein in mitotic cell extracts. These data support models by which DYNLT3 links cytoplasmic dynein to Bub3 containing spindle
checkpoint complexes and contributes to checkpoint complex function during congression and/or during the inactivation of the checkpoint by transporting the complexes from kinetochores to spindle poles.
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FOOTNOTES

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1. Cytoplasmic dynein 1 light chain subunit nomenclature. There are three functionally distinct light chain families in the cytoplasmic dynein complex and each family has at least two members (or isoforms). The names of the light chains all begin with DYN for dynein, followed by L for light chain, then additional letters that designate the families. The family names are based on the old common name of the first identified member of each family: DYNLT (T for the Tctex1 family); DYNLRB (RB for the Roadblock family); and DYNLL (L for the LC8 family). The different members (or isoforms) of the families are distinguished by adding numbers to the name, for example DYNLT1 and DYNLT3 are the two members of the DYNLT light chain family. In the text, a light chain polypeptide subunit is identified at first mention with its formal name followed by the old common name in parentheses, for example DYNLT3 (previously called rp3). This nomenclature has been endorsed by the Human Genome Organization Nomenclature Committee (HGNC) and the International Committee on Standardized Nomenclature for Mice (2)

2. The abbreviations used are: 3-AT, 3-amino-1,2,4-triazole; DAPI, 4’,6-diamidino-2-phenylindole; GFP, green fluorescent protein; GSH, glutathione-Sepharose; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVDF, polyvinyl fluoride; RT-PCR, reverse transcriptase polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; siRNA, small interfering RNA.

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FIGURE LEGENDS

Figure 1. The cytoplasmic dynein light chain, DYNLT3, binds to the spindle checkpoint protein Bub3. A. Yeast two-hybrid assay. Yeast transformants expressing the indicated constructs were grown on plates with (+His) or without histidine (-His) and the indicated concentrations of the histidine biosynthesis inhibitor 3-AT. Only those co-transformants expressing interacting constructs were able to grow in the absence of histidine and in the presence of 3-AT. DYNLT3 and human Bub3 are in the pGBK7 and pGAD7 vectors respectively. B. β-Galactosidase Expression Assay. The positive interaction of DYNLT3 and Bub3 was confirmed with a semi-quantitative β-galactosidase assay (+++), substantial growth, (+) marginal growth, (-) no growth. C. In vitro Binding Assay. Bacterial lysates expressing His-DYNLT3 were incubated in a pull-down assay with either GST-Bub3NT or GST, bound to GSH beads. Right Panel. His-DYNLT3 bound to GST-Bub3NT beads but not to GST control beads. His-DYNLT3 was detected with Western blots using anti-His antibody. The input lane shows 2% of the His-DYNLT3 used in the pull-down assays (1.5 ml per reaction) and the experimental lanes were loaded with 50% of the pellets. Left Panel. Coomassie Blue stained SDS-PAGE demonstrates the relative concentrations and purity of the GST-Bub3NT (left lane) and GST (right lane) used in the assays.

Figure 2. DYNLT3 is the only dynein light chain that binds the Bub3 checkpoint protein. Lysates of COS7 cells expressing either myc-DYNLT3, -DYNLT1, -DYNLL1, -DYNLL2, -DYNLRB1, -DYNLRB2 were incubated with GST-Bub3 or GST, bound to GSH-Sepharose beads. The lysates and proteins eluted from the beads were resolved by SDS-PAGE and Western blotted to PVDF. The membrane was probed with an antibody to the myc epitope. A. Bub3 binds only to the DYNLT3 light chain not to the DYNLT1, DYNLL1, DYNLL2, DYNLRB1, or DYNLRB2 light chains. The lanes labeled “Inputs” demonstrate the successful expression of the transfected proteins used in the each pull-down assay and represent 50% of the over-expressed proteins used in the pull-down assay (10 μl per reaction) and the experimental lanes were loaded with 50% of the pellets. The "UT" lane shows untransfected cell lysates. B. Coomassie Blue stained gel showing the relative concentrations and purity of the GST-Bub3 and GST proteins (identified with arrow heads on the left) used in each pull-down reaction. The molecular weight markers are shown on the right. The concentration of GST used as a negative control was greater than the GST-Bub3 concentration.

Figure 3. Bub3 interacts with the cytoplasmic dynein complex. A. The dynein complex co-purifies with GST-Bub3. Lysates of mitotic NRK cells (mit. NRK) were incubated with GST-Bub3 or GST alone, bound to GSH beads. Bound proteins were eluted and analyzed by SDS-PAGE and Western blotting. On the left side of the panels are the antibodies used to screen for three dynein subunits; HC heavy chain, IC intermediate chain, LC light chain. The three representative dynein subunits bound to the GST-Bub3 but not to GST protein alone. The "Input" lane shows the subunits present in the cell lysates and it represents 20% of the proteins used in the pull-down experiments (100 μl per reaction) and the experimental lanes were loaded with 50% of the pellets. B. Bub3 and Mad2 co-immunoprecipitate with cytoplasmic dynein. Cytoplasmic dynein was immunoprecipitated from mitotic (mit., upper set) or interphase (inter., lowe set) NRK cell lysates with the 74.1 antibody. The immunoprecipitates were resolved by SDS-PAGE and Western blotted to PVDF, and the blots were probed with antibodies to the dynein intermediate chain, Bub3, and Mad2 as indicated. The "Input" lane shows the subunits present in the cell lysates and it represents 10% of the proteins used in the pull-down experiments (100 μl per reaction) and the experimental lanes were loaded with 50% of the pellets.
the pellets. The Mad2 levels in the interphase NRK cells were too low for analysis [data not shown and (58)].

Figure 4. Comparison of the mitotic distributions of the DYNLT3 dynein light chain with the CREST kinetochore antigen and Bub3.

A. **DYNLT3 co-localizes with Crest at prometaphase kinetochores.** Comparison of distribution of DYNLT3-GFP fluorescence (green) with antibody staining for CREST antigen (red) or Bub3 (red) treated LLCPK cells transfected with DYNLT3-GFP and processed for immunocytochemistry. The two overlays show DYNLT3 and either Crest or Bub3 (as indicated) with and without DAPI (blue) to show chromatin.

B. **DYNLT3 does not co-localize with the Crest antigen in later mitotic stages.** Comparison of distribution of DYNLT3-GFP fluorescence (green) with antibody staining for CREST antigen (red) and chromosomes (DAPI, blue) in LLCPK cells. The overlay contains the three images. Image sets from the mitotic stages indicated on the left; metaphase, anaphase and telophase.

C. **Comparison of the distributions of DYNLT3 and tubulin in metaphase.** Comparison of distribution of DYNLT3-GFP fluorescence (green) with antibody staining for tubulin (red), and chromatin (DAPI, blue). The overlay shows the three color images superimposed.

D. **DYNLT3 and Bub3 do not co-localize at later mitotic stages.** Comparison of distribution of DYNLT3-GFP fluorescence (green) with antibody staining for Bub3 (red) chromosomes (DAPI, blue) and the three color overlay. Image sets are from the mitotic stages indicated on the left, metaphase and anaphase. Scale bars are 10 μm.

Figure 5. Effects of DYNLT3 siRNA on mitotic progression. NRK cells were co-transfected with a reporter GFP vector and either DYNLT3 siRNA or control siRNA.

A. **Knock down of DYNLT3 protein levels.** Cells were lysed 48 h after transfection, analysed by SDS-PAGE and Western blotting, and the blot was probed with antibodies to tubulin and DYNLT3.

B. **Effect of DYNLT3 knockdown on mitotic index.** Cells were fixed 48 h after transfection, processed for immunocytochemistry with an antibody to tubulin and DAPI for chromatin. Cells were scored as either interphase or mitotic based on chromosome and microtubule configurations. Means and S.E. were computed. The differences between the siRNA and control siRNA was significant at p<0.002, Student’s t test.

C. **Effect of DYNLT3 knockdown on progression through mitosis.** Mitotic cells were further scored for position in the cell cycle; P/PM, prophase/prometaphase; M, metaphase; A, anaphase, and T, telophase. All values are means from six interations (an average of 6,364 cells/ iteration). The relative number of cells in each mitotic stage is shown. For ease of reference, the control transfected prophase/prometaphase value (0.3% of total cells) was set at 1 and the other values scaled accordingly. Means and S.E. were computed. The difference between the prophase/ prometaphase data was significant at p<0.0002, Student’s t test.
**A**

| Plasmids     | DYNLT3 | pGBKT7 | Bub3 | pGADT7 |
|--------------|--------|--------|------|--------|
| + His        |        |        |      |        |
| - His        |        |        |      |        |
| Selection    |        |        |      |        |
| Plates       |        |        |      |        |
| - His        |        |        |      |        |
| - His        |        |        |      |        |
| - His        |        |        |      |        |
| - His        |        |        |      |        |
| + His        |        |        |      |        |

| Concentration |          |          |
|---------------|----------|----------|
| 0 mM          |          |          |
| 5 mM [3-AT]   |          |          |
| 10 mM         |          |          |
| 15 mM         |          |          |
| 25 mM         |          |          |

**B**

| Bait       | Target | Bub3 | pGADT7 |
|------------|--------|------|--------|
| DYNLT3     | +++    | +    |        |
| pGBKT7     | (-)    | (-)  |        |

**C**

- GST-Bub3NT
- GST

Coomassie Blue

Lo et al., Figure 1.
A

|          | Inputs | UT  | GST Bub3 | GST | GST Bub3 | GST |
|----------|--------|-----|----------|-----|----------|-----|
| MycDYNL3 | +      | +   | +        | +   | +        | +   |
| MycDYNL1 | +      | +   |           | +   | +        | +   |
| MycDYNL2 | +      | +   | +        | +   | +        | +   |
| MycDYNL1 | +      | +   | +        | +   | +        | +   |
| MycDYNL2 | +      | +   | +        | +   | +        | +   |

Blot: Anti-myc

B

GST-Bub3

GST

Coomassie Blue

kDa

93
49.8
35.8
29

Lo et al., Figure 2.
A

| GST-Bub3 + mit. NRK | GST + mit. NRK | Input |
|---------------------|----------------|-------|
| Blot: HC-8          | Dynein HC      |       |
| Blot: 74.1          | Dynein IC      |       |
| Blot: R1            | Dynein DYNLT3 LC |      |

B

| Input | IP: mit. NRK | Beads | Dynein |
|-------|--------------|-------|--------|
| Blot: 74.1 | Dynein IC |       |        |
| Blot: anti mBub3 | Bub3 |       |        |
| Blot: anti Mad2 | Mad2 |       |        |

| Input | IP: inter. NRK | Beads | Dynein |
|-------|----------------|-------|--------|
| Blot: 74.1 | Dynein IC |       |        |
| Blot: anti mBub3 | Bub3 |       |        |

Lo et al., Figure 3.
Prometaphase

Lo et al., Figure 4A
| Phase   | Crest | DYNLT3 | DAPI |
|---------|-------|--------|------|
| Metaphase | ![Crest](image1) | ![DYNLT3](image2) | ![DAPI](image3) |
| Anaphase | ![Crest](image4) | ![DYNLT3](image5) | ![DAPI](image6) |
| Telophase| ![Crest](image7) | ![DYNLT3](image8) | ![DAPI](image9) |

Lo et al., Figure 4B
Lo et al., Figure 4C
Lo et al., Figure 4D
Lo et al., Figure 5.
The DYNLT3 light chain directly links cytoplasmic dynein to a spindle checkpoint protein, Bub3

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