You Keep Me Hangin' On: Dynactin's p24 is Essential for Microtubule Anchoring

By:

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Nicholas J. Quintyne, and has been approved by the members of his supervisory committee. It was submitted to the faculty of The Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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Abstract

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Dynactin is a multisubunit protein complex that functions as a processivity cofactor to cytoplasmic dynein, assisting in vesicle transport and cell division. Independent of dynein, dynactin also serves to anchor microtubules to the centrosome. The functions of the majority of dynactin's subunits have been described to a certain degree; however, the p24 subunit remains largely uncharacterized. Among the few things that are known about p24 are that it has a predicted molecular weight of about 20,822 Da, forms an α-helix, and binds directly to the $p150^{Glued}$ subunit. In order to explore its function further, we have performed shRNA-mediated knockdown, and fluorescent microscopy. We observe that microtubule disorganization is amplified due to the loss of p24. Our findings support the model that p24 serves as reinforcement to stabilize $p150^{Glued}$ at the centrosome.

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Kasoy: for being so handsome

For the warmth in our hearts, Carol Sun

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Introduction

A diverse variety of processes must be carried out within cells in order to maintain the integrity of organisms. The cytoskeleton, a dense network of different filaments, is required for many of these vital functions which include governing intracellular trafficking, maintaining cellular shape, and allowing for cell division (Mays et al., 1994). Among the filamentous components of the cytoskeleton, is the microtubule network (Figure 1) which has important roles in cargo transport and mitosis (Inoue & Sato, 1967; Vale & Hotani, 1988).

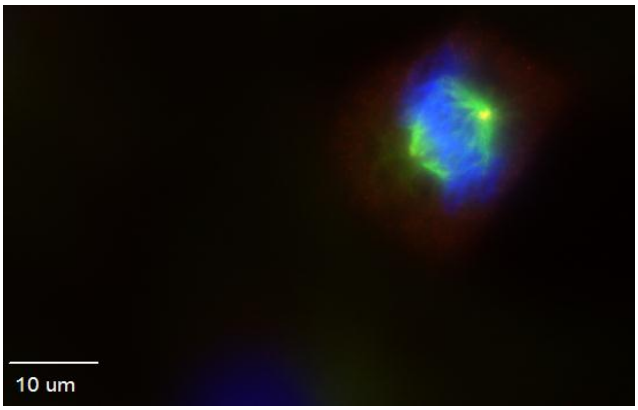


Figure 1: Cell in metaphase stage of mitosis. Microtubules (green) attached to DNA (blue). Bar = 10μm.

Microtubules organize in astral arrays that radiate from the centrosome in a polarized fashion and use the motor proteins kinesin and dynein (Figure 2) to carry out specific objectives such as intracellular transport of proteins and organelles (Amos & Klug, 1974; Hirokawa, 1998). These motor proteins require the hydrolysis of ATP as an energy source in order to move along the microtubules efficiently and transport cargo (Vale et al., 1985; Paschal et al., 1987). However, dynein requires the function of another protein complex, dynactin, for optimal efficiency (Schroer & Sheetz, 1991; Gill et al., 1991; King and Schroer, 2000).

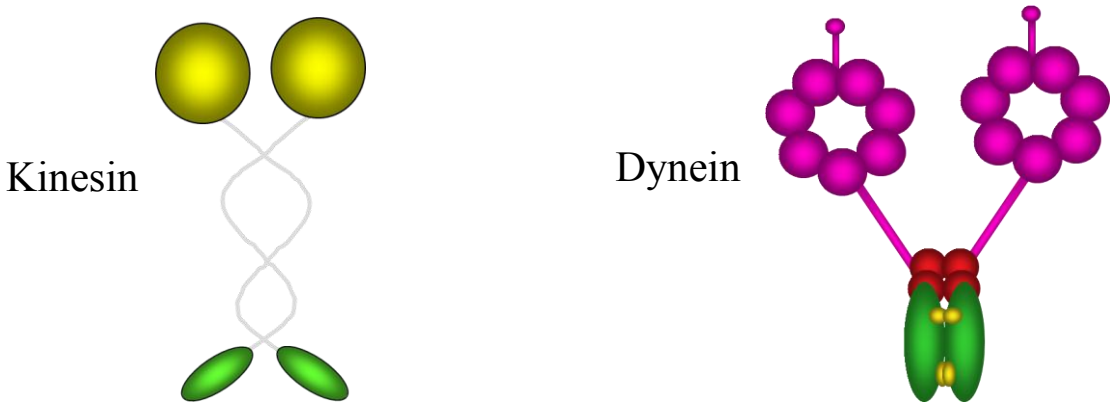


Figure 2: Models of the motor proteins: 'typical' homodimer kinesin shown left, where the yellow heads are ATPase/MT binding sites and the green tails are cargo binding sites, with a coiled stalk binding the two domains together; and cytoplasmic dynein shown right, where the purple are the heavy chains, red are the light intermediate chains, green are the intermediate chains, and yellow are the light chains.

Dynactin (Figure 3) was initially an unidentified cytosolic factor that served as an activator to dynein during long range vesicle transport along microtubules (Schroer & Sheetz, 1991). Dynactin was successfully isolated, then analyzed to reveal that the cofactor is an asymmetric multisubunit protein complex with two separate subcomplexes, that colocalizes with dynein to enhance processivity during vesicle movement and mediate dynein-based cargo binding (Gill et al., 1991; Schafer et al, 1994a; Gaglio et al., 1997; King & Schroer, 2000; Quintyne & Schroer 2002). Although dynactin is essential for dynein function, dynactin has other important functions independent of dynein such as anchoring microtubule minus-ends and preserving microtubule organization (Quintyne et al., 1999; Quintyne and Schroer, 2002).

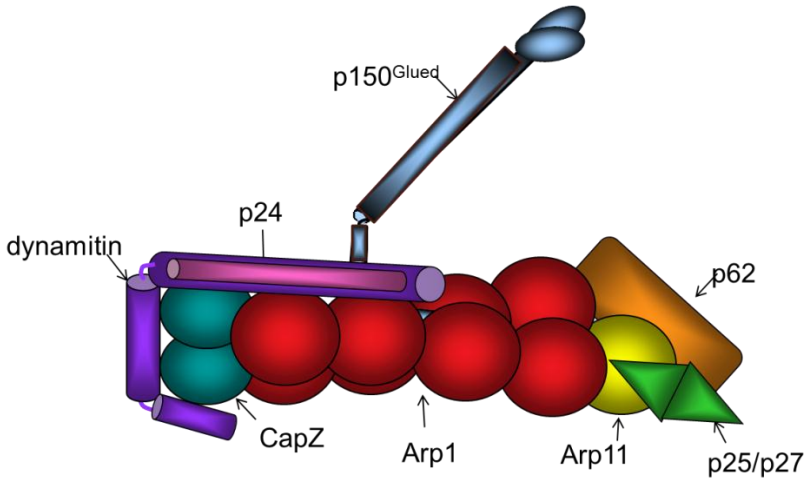


Figure 3: Model of dynactin with subunits depicted based on deep-etch electron microscopy, immunoblotting and SDS page. Dynactin is composed of two subdomains: the shoulder/sidearm complex comprised of $p150^{Glued}$, p24, and dynamitin and the actin-related minifilament backbone which is comprised of CapZ, Arp 1, Arp11, p25, p27, and p62.

It has been found that dynactin is enriched at the centrosome where it functions in microtubule anchoring (Clark & Meyer, 1992; Quintyne et al., 1999). Microtubules are dynamic filaments composed of alternating α-tubulin and β-tubulin protein monomers collectively referred to as protofilaments, which continuously associate and dissociate from a main, hollow tube (Kirschner, 1980; Ludeuna, 1997). Microtubules originate from the centrosome in a polarized manner, with plus-ends radiating towards the cell periphery and minus-ends that nucleate from a structure found at the centrosome that contains a third type of tubulin, γ-tubulin, which is a component of the γ-tubulin ring complex known as γ-TuRC (Amos & Klug, 1974; Zheng et al., 1995). The γ-TuRC consists of different proteins capable of capping the minus-ends, thus allowing for the microtubules to cluster their minus-ends at the centrosome and polymerize their plus-ends towards the cell periphery (Zheng et al., 1995). The maintenance and organization of the

microtubules is achieved by a plethora of different proteins found in this region of the centrosome, referred to as the microtubule-organizing center (MTOC; Dictenberg, 1998), which contains a discrete anchoring complex consisting of several proteins including dynactin (Quintyne et al., 1999), ninein (Mogensen et al., 2000), Cep135 (Ohta et al., 2002) and PCM-1 (Dammerman & Merdes, 2002). However, the manner in which these proteins interact together remains uncertain. The functional integrities of the centrosome as the MTOC and its associated proteins have proven to be essential for imperative functions such as timely progression through the cell cycle and microtubule organization (Quintyne et al., 1999; Quintyne & Schroer, 2002).

The dynactin complex is comprised of two distinct subdomains: the protruding shoulder-sidearm region containing dynamitin, $p150^{Glued}$, and p24; and the minifilament, actin-related backbone domain containing CapZ, Arp 1, Arp11, p25, p27, and p62 (Eckley et al., 1999). The general properties and functions of most of these proteins have been investigated, yet there is still much to be explored. The dynamitin subunit, which binds to both p24 and $p150^{Glued}$, is believed to be responsible for preserving the structural integrity of the complex (Echeverri et al., 1996; Eckley et al., 1999; Quintyne et al., 1999; Melkonian et al., 2007). Overexpression of dynamitin results in the deterioration of the dynactin structure, disorganization of spindle poles, delay in mitosis, and inefficient endomembrane motility (Echeverri et al., 1996; Burkhardt et al., 1997; Eckley et al., 1999; Quintyne et al., 1999; Valetti et al., 1999; Melkonian et al., 2007). The $p150^{Glued}$ subunit, the largest component that projects from the complex, functions to mediate motor and microtubule binding and increase the efficiency of motor processivity (Karki

& Holzbaur, 1995; Waterman-Storer et al., 1995; King & Schroer, 2000). $p150^{Glued}$ contains two microtubule binding domains with different affinities: a CAP-Gly (cytoskeleton-associated, Glycine-rich) motif at the N-terminus that binds tightly to microtubules, inhibiting dynein motility and a basic domain which binds loosely to microtubules, increasing dynein processivity four-fold in a movement analogous to "skating" along microtubules (Culver-Hanlon et al., 2006). However, in order for $p150^{Glued}$ to carry out these functions correctly, the other dynactin subunits must be present and functional (McGrail et al., 1995). The C-terminus of the $p150^{Glued}$ subunit has been proposed to bind directly to the Arp1 component of the minifilament backbone in dynactin, which as a whole functional unit is capable of binding to cargo (Waterman-Storer et al., 1995; Holleran et al., 1996). The Arp1 subunit contains conserved sequences that are similar to conventional actin, and thus is able to use ATP hydrolysis as an energy source to polymerize short filaments (Bingham & Schroer, 1999). Another component of the minifilament backbone, Arp11, also contains sequences homologous to actin (Eckley et al., 1999). However, as opposed to Arp1, the sequence of Arp11 indicates that interaction will take place only between Arp1 or actin filaments with pointed ends (Eckley et al., 1999). This restriction will not allow for polymerization of filaments, but instead be used to prevent further Arp1 polymerization (Eckley et al., 1999). At one end of the minifilament backbone lies the subunit CapZ, another protein with actin homology that serves as a barbed-end capping protein (Schafer et al., 1994a; Schafer et al., 1994b). At the other end of the backbone is a collection of different subunits referred to as the pointed end complex, containing subunits p62, p25, and p27, which is found attached to

Arp11 (Eckley et al., 1999). The sequences from the pointed end complex contain predicted cargo-binding motifs, where estimates based on biochemical properties suggest the complex may also function in membrane binding (Eckley et al., 1999). The subunits of the actin-related, minifilament backbone are more highly conserved than the shoulder-sidearm component (Eckley & Schroer, 2003). The precise role of the p24 subunit of the dynactin complex remains to be the only component that is largely uncharacterized.

Minimal data exist about p24. It has a predicted weight of about 20,822 Daltons, is found only in dynactin, shares no sequence similarities with any other enzymes or proteins, forms an α-helix, and binds directly to the $p150^{Glued}$ subunit (Pfister et al., 1998; Karki et al., 1998). It is possible that p24 is involved in regulating microtubule anchoring at the centrosome or recruitment of centrosomal structural components, among other possibilities. Previous research has been conducted in order to find its function. Overexpression of p24 had no effect on dynactin structural integrity, unlike dynamitin overexpression which destroyed the complex. Thus, p24 most likely does not function to preserve structural integrity of the dynactin complex (Quintyne et al., 1999). However, p24 overexpression results in microtubule disorganization, where the wildtype phenotype of microtubules forming astral arrays radiating from the centrosome is decreased (Quintyne et al., 1999; McCullough, 2011). These data indicate that p24 may function in microtubule anchoring and centrosome organization (Quintyne et al., 1999). In the same study, loss of microtubule organization was seen when the other two shoulder-sidearm subunits, dynamitin and $p150^{Glued}$ as well as sub-regions of $p150^{Glued}$ were overexpressed. Due to the observation from previous studies that the dynein-dynactin complex is

required for correct Golgi localization (Burkhardt et al., 1997), it was imperative to test whether p24 played a role in mediating this localization. When p24 was overexpressed, Golgi localization remained intact, indicating that p24 does not likely play a role in positioning of membranes and organelles (Quintyne et al., 1999; McCullough, 2011). Unlike p24, when the other components of the shoulder-sidearm subcomplex, dynamitin and $p150^{Glued}$, are overexpressed, Golgi integrity is compromised (Quintyne et al., 1999). These findings indicate that p24 has the role of anchoring microtubules independent of the other shoulder-sidearm subunit-dependent functions. Although p24 overexpression does not affect Golgi localization, it leads to the loss of $p150^{Glued}$ at the centrosome, while the Arp1 subunit remains (Quintyne et al., 1999). This finding shows that the dynactin complex begins to degrade due to p24's overexpression (Quintyne et al., 1999), but only at the centrosome. Despite that p24 overexpression leads to the loss of $p150^{Glued}$, which binds to microtubules, all of the components of the shoulder-sidearm subunit have shown to exhibit the same microtubule organization phenotype when overexpressed, indicating that each subunit has a role in focusing microtubules (Quintyne et al., 1999). These data in conjunction with the results from the sedimentation assays, which showed that the dynactin structure remained intact despite p24 overexpression, indicate that dynactin at the centrosome, where $p150^{Glued}$ is lost, exists in a different shape as opposed to when it is in the cytoplasm. Knockdown of p24 resulted disorganization of microtubules, though the extent of the disorganization was not as severe as overexpression, and an increase in cells with abnormal amounts of γ-tubulin foci at the centrosome (McCullough, 2011). Overexpression of p24 also leads to the apoptosis of cells before they enter mitosis

(Quintyne & Schroer, 2002). Experimentation producing this result was done with different populations of cells consisting of those at around the same point of the cell cycle, and those at random points of the cell cycle. Although cells do not enter mitosis, DNA replication and centrosome duplication were unaffected (Quintyne & Schroer, 2002). These findings suggest that p24 may function in the regulation of the cell cycle.

For my thesis project, I performed knockdown experiments on p24 and used indirect immunofluorescence in order to observe the phenotype of microtubule arrangement, the number of γ-tubulin foci at the centrosome, the localization of the Golgi complex, and calculate the mitotic index. The loss of p24 resulted in cells with a normal mitotic index, and correct localization of the Golgi, but extremely disorganized microtubules, and a slight increase in cells with abnormal amounts of γ-tubulin foci. I also performed microtubule regrowth assays on p24 knockdown cells and used indirect immunofluorescence afterwards to analyze the resulting effects on microtubules and γ-tubulin. Although the regrowth assay was successfully carried out in control cells, the protocol performed with p24 knockdown cells led to inconsistent depolymerization. Based on these findings, we propose that p24 acts as a facilitator, maintains the functional integrity of dynactin, and allows for functional microtubule anchoring at the centrosome.

Methods

Tissue Culture:
COS-7 (ATCC, Manassas, VA) cells were grown using Dulbecco's Modified Eagle Medium (Sigma Chemical Company, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (Sigma) and Penicillin-Streptomycin (MP Biomedicals, Solon, OH). Cells were incubated at 37°C in 5% CO_2. The cells were passaged with 0.05% Trypsin-EDTA (Sigma) when confluency was greater than 80%.

Plasmids:

Combinations of shRNA plasmids pLKO.1-puro from the set NM_007234 (Sigma) was used to knockdown the gene expression of p24 using the following sequences:
5'-CCGGCTTTGTTACAAGGCAGAGGAACTCGAGTTCCTCTGCCTTGTAACAA AGTTTTTG-3',
5'-CCGGGATCTGATCAAGTACCTGGATCTCGAGATCCAGGTACTTGATCAGA TCTTTTTG-3',
5'-CCGGCAATGCTTCTCTCCAAGCAATCTCGAGATTGCTTGGAGAGAAGCAT TGTTTTTG-3'.

Antibodies:

The following primary antibodies were used: DM1A (mouse) anti-α-tubulin (1:200 dilution, Sigma), rabbit anti-γ-tubulin (1:500 dilution, Sigma), rabbit anti-Giantin (1:1000 dilution, ABCAM, Cambridge, MA), and mouse anti-p150 (1:100 dilution, BD

Biosciences, Rockville, MD). The secondary antibodies used were Texas-Red-X anti-mouse or anti-rabbit (1:250 dilution, Invitrogen, Carlsbad, CA) or Alexa 488 conjugated goat anti-mouse or anti-rabbit (1:250 dilution, Invitrogen).

Lipid Transfection:

3µl of FuGene 6 (Roche Diagnostics, Indianapolis, IN) was added to 97µl of Opti-MEM (Gibco, Grand Island, NY). After an incubation period of 5 minutes at room temperature, 3µl of shRNA plasmid was added to the solution. After incubating at room temperature for 15 minutes, the solution was added dropwise to coverslips. The cells were incubated for 72 hours after transfection.

Microtubule Regrowth Assay:

After the coverslips were washed with PBS, 2 mL of media-containing 33µM Nocodazole (Nz; Sigma) were added to each well and the coverslips incubated on ice for 30 minutes to allow for the depolymerization of MTs. The Nz-containing media was aspirated, then the coverslips were washed five times with 2mL of regular media per well at room temperature. The coverslips incubated at 37°C to allow for regrowth. The coverslips were fixed with methanol at -20°C at the time intervals of 0, 5, 10, 15, 20, 25, 30, and 60 minutes

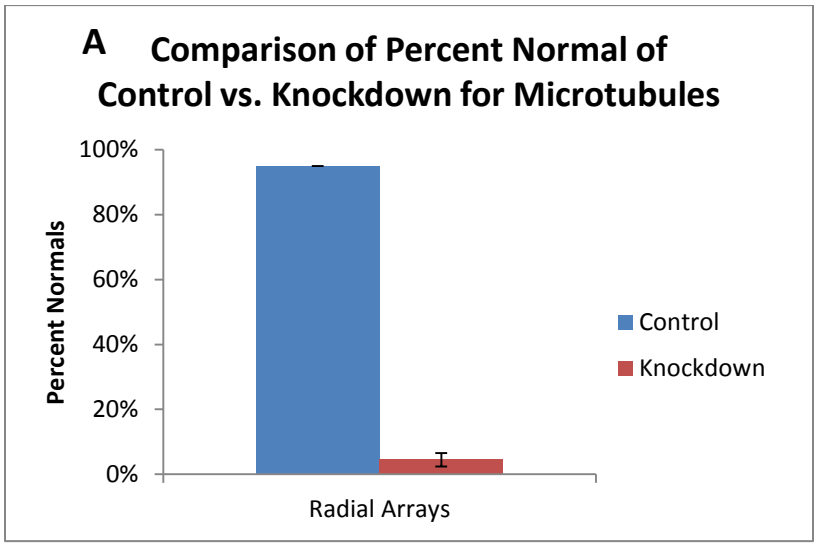
Immunofluorescence:

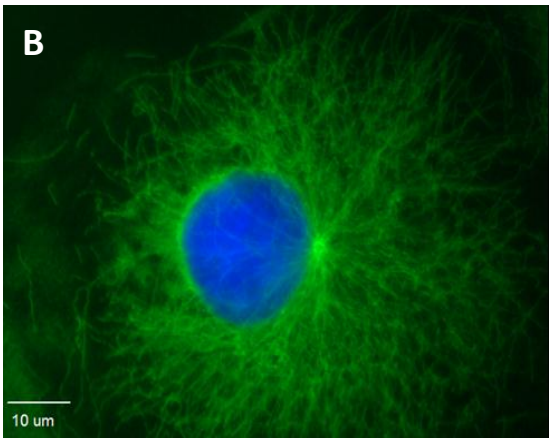
Cells were grown on 22mm^2 coverslips in six-well culture plates to a density of about 2 x 10^5 cells. The media from the plates were aspirated and the coverslips were washed with PBS then aspirated. Methanol was added to the coverslips at -20°C, then placed in the freezer for 5 minutes. After the coverslips were removed from the freezer, the methanol was aspirated and 1.5 mL of PBST/BSA (1.5%) was added to each well and allowed to incubate at room temperature for 15 minutes then aspirated. Each coverslip was then given 150µl of primary antibody that was diluted in PBST/ BSA and allowed to incubate at room temperature for 30 minutes. The primary antibody solution was then aspirated from the coverslips, which were then washed three times with PBS for three to five minutes each while aspirating between each wash. After the last PBS wash, each coverslip was given 150µl of secondary antibody that was also diluted in PBST/BSA and incubated at room temperature for 15 minutes. The secondary antibody solution was then aspirated and the coverslips were washed three times with PBS for three to five minutes each while aspirating between each wash. After the last wash, 100µl of 4',6-diamidino-2-phenylindole (DAPI, Sigma) was added to each coverslip and incubated at room temperature for 30 seconds, then aspirated. The coverslips were then washed three times with water for 30 seconds each, while aspirating after only the first two washes. While still in the water from the last water wash, the coverslips were mounted on slides using mounting medium made of p-phenylenediamine (Ultra, North Kingston, RI) in glycerol. After the mounted coverslips were allowed to dry for 5 minutes, the edges were sealed with nail polished and the slides were stored at -20°C. In order to analyze the

immunostained cells, an Olympus IX-81 Inverted Fluorescence Microscope with a 100X 1.65 N.A. objective (Olympus America, Center Valley, PA) was used while Hamamatsu C4742-95 CCD camera (Hamamatsu Corporation, NJ) was used to capture images. During analysis, the Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO) ran simultaneously, which was the same software used to manipulate and enhance the images. About 300-500 cells were analyzed per slide and all experiments were carried at least twice.

Results

According to sedimentation assays, immunoblotting, and electron microscope analysis, dynactin is a multi-protein complex that is involved microtubule anchoring (Gill et al., 1991; Quintyne et al., 1999). In order to gain further understanding of the dynactin complex, it is necessary to distinguish the functions of its subunits and the roles they contribute to the overall function. The functions of most of the subunits have been at least partially explored (Schroer, 2005). However, the function of the p24 subunit remains undefined. Previous research has shown that p24 overexpression leads to several cellular defects, including the loss of the radial array and subsequently microtubule disorganization (Quintyne et al., 1999). Based off this finding, it was important to continue to manipulate the expression of p24 in the attempt to find its function. Initially, the effects of shRNA-mediated knockdown of p24 on microtubule organization were analyzed (Figure 4). Under normal conditions, microtubules radiate outwards in a radial array from the centrosome (Figure 4B). In our control cell population, about 96% had organized microtubules radiating from the centrosome (Figure 4A). On the other hand, when p24 was knocked down, only about 5% of cells had organized microtubules, displaying disorganization with microtubule filaments scattered throughout the cell (Figure 4C).





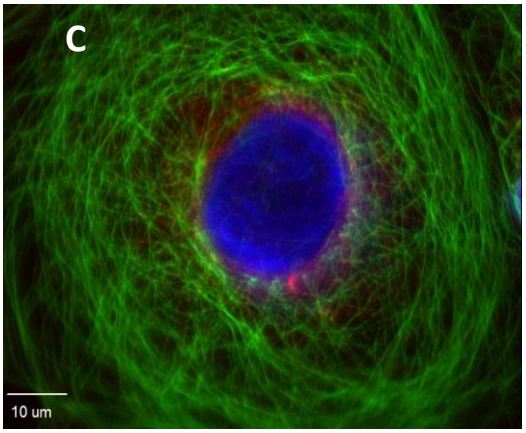
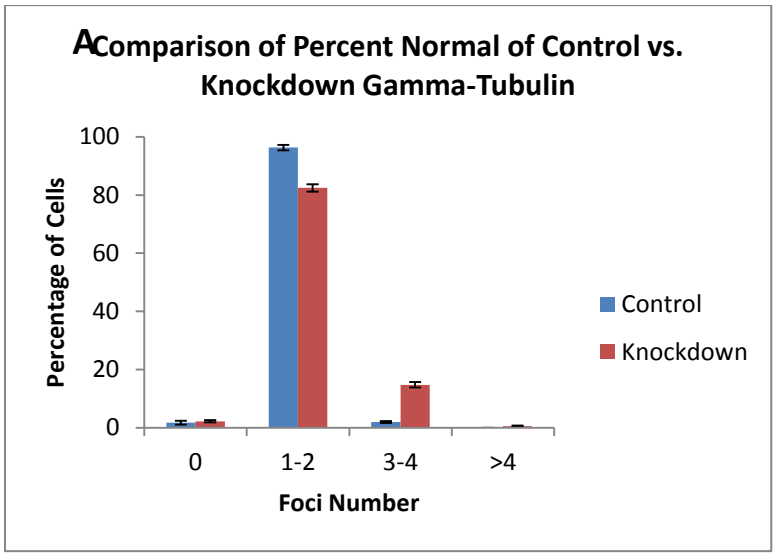


Figure 4: Analysis of microtubule organization before and after knockdown of p24 expression. Cells were characterized as normal when microtubules were seen radiating from the centrosome. (A) Microtubule organization quantified in control cells and p24 knockdown cells. (B) Control (C) p24 knockdown. Microtubules: green, DAPI: blue, and Golgi: red. Bar = 10 µm.

Afterwards, γ-tubulin organization was analyzed and quantified in control cells and p24 knockdown cells (Figure 5). Centrosomal organization of γ-tubulin is characterized in normal cells as having 1-2 foci near the nucleus. Foci numbers that deviate from this quantity are considered abnormal. In our control cell population, about 96% of cells had a normal frequency of 1-2 foci. When p24 was knocked down, this value decreased to about 82% of cells with normal foci, and the number of aberrant cells with more than 2 foci increased to about 18%.



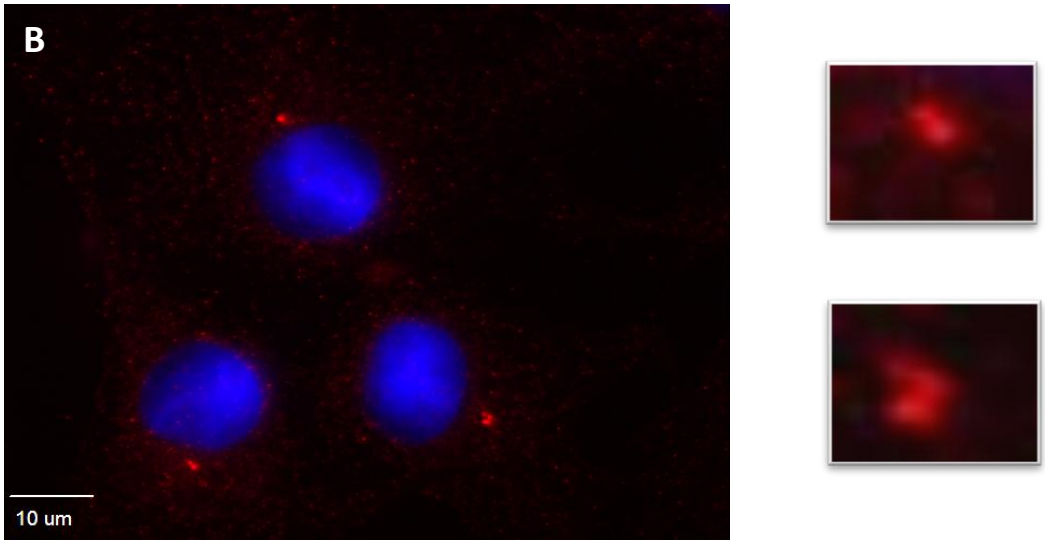


Figure 5: Analysis of γ-tubulin foci number at the centrosome. Normal cells are characterized as having 1-2 foci. (A) γ-tubulin foci quantified for control cells and p24 knockdown cells. (B) Control cells with 1-2 foci and a p24 knockdown cell with 4 foci and accompanying insets. γ-tubulin: red and DAPI: blue. Bar = 10 μm.

Previous studies have shown that p24 overexpression has no effect on Golgi localization (Quintyne et al., 1999). As a result, the effects of p24 knockdown on Golgi localization were observed (Figure 6). Our findings show that p24 knockdown also had no effect on Golgi organization and localization, where no difference from control cells was detected.

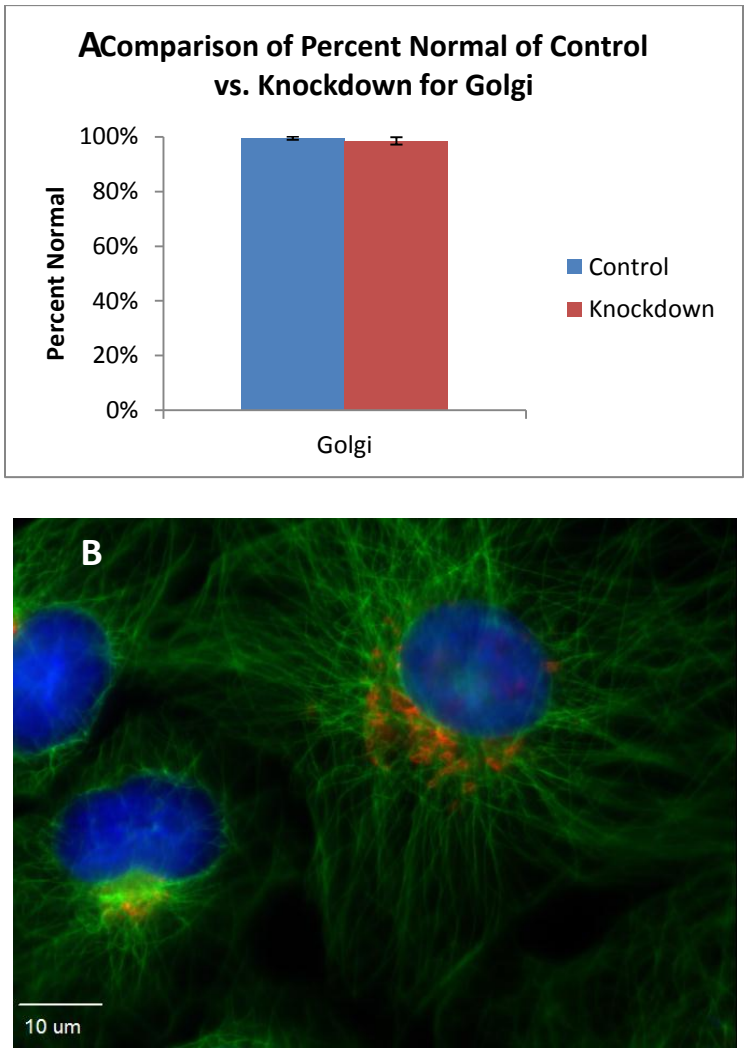
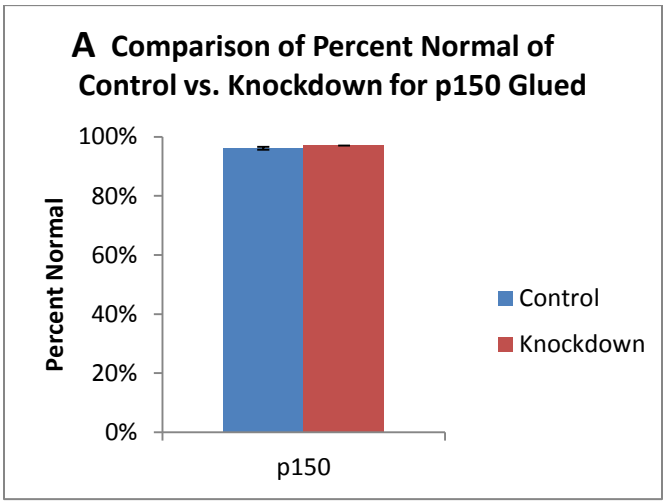


Figure 6: Golgi organization after p24 knockdown. (A) Quantification of Golgi organization (B) Cells with correctly localized and intact Golgi after p24 knockdown. Giantin: red, microtubules: green, and DAPI: blue. Bar = 10 µm.

Next, the effect of p24 knockdown on the structural integrity of the dynactin complex was analyzed. This was done by staining for the $p150^{Glued}$ subunit of dynactin in control cells and in p24 knockdown cells (Figure 7). Previous research has shown that dynactin is normally enriched at the centrosome but when p24 was overexpressed, about 65% of cells had $p150^{Glued}$ correctly localized at the centrosome (Clark and Meyer, 1992; Quintyne et al., 1999). When we knocked down p24, however, no difference was seen between the knockdown population and the control population (Figure 7A). The $p150^{Glued}$ subunit remained localized at the centrosome (Figure 7B).



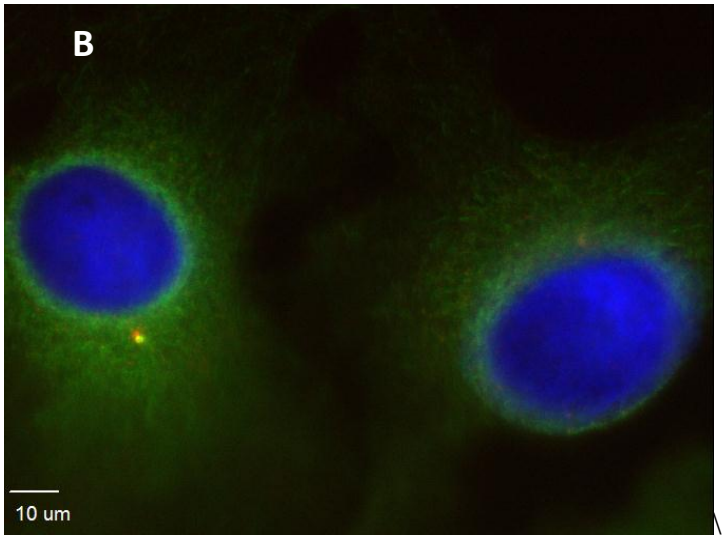


Figure 7: Centrosomal localization of the p150^{Glued} subunit of the dynactin complex. (A) Quantification of p150^{Glued} localization at the centrosome (B) Cell with correct p150^{Glued} localization (left) and cell with p150^{Glued} lost from the centrosome. p150^{Glued}: green and DAPI: blue. Bar = 10 µm.

Due to the clear increase in abnormal microtubule structures, the possible effects of p24 knockdown on microtubule dynamics were analyzed. Using microtubule regrowth assays, it is possible to observe microtubule dynamics and subsequent organization overtime. This was carried out successfully in control cells (Figure 8): immediately after nocodazole treatment to induce depolymerization of the microtubules at t = 0 min, no asters were visible and free-floating tubulin proteins were seen dispersed throughout the cell. As time continued, small asters began to appear and at a more frequent rate, until full microtubules radiated from the centrosome and appeared as normal, fully polymerized microtubules once more. However, when the analysis was performed in p24 knockdown cells, inconsistent rates of depolymerization, even at higher concentrations of nocodazole, were observed making it impossible to assay regrowth under the same conditions used for controls.

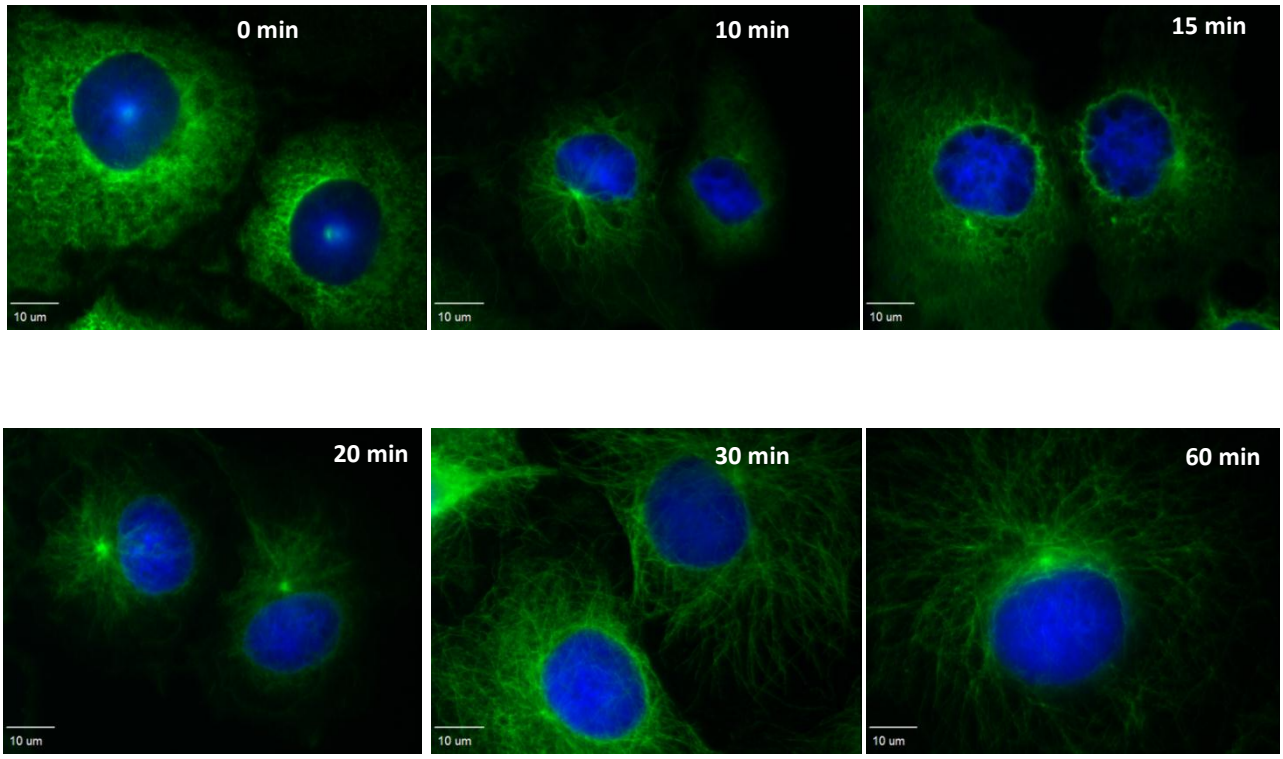


Figure 8: Control cells undergoing microtubule regrowth overtime after depolymerization with nocodazole. Microtubules: green and DAPI: blue. Bar = 10 µm.

Discussion

The purpose of my research was to identify the function of the p24 subunit of dynactin. In addition to exploring p24's function further, our research has allowed us to gain understanding about the shoulder/sidearm complex as well. Our findings show that p24 may serve a role in microtubule anchoring at the centrosome. Previous research has shown that overexpression of the subunits of the shoulder/sidearm complex leads to the loss of microtubule organization (Quintyne et al., 1999). It was believed that overexpression of each of the three subunits affected microtubule binding in different ways. Overexpression of the $p150^{Glued}$ subunit results in large amounts of the protein, which actively compete for binding sites with microtubules, thus inhibiting endogenous dynactin and cytoplasmic dynactin from binding to microtubules. Overexpression of dynamitin led to the dissociation of the dynactin complex into the shoulder/sidearm and minifilament components (Echeverri et a., 1996; Quintyne et al., 1999; Melkonian et al., 2007), resulting in no remaining dynactin to perform its function at the centrosome. Previous results from overexpressing p24 led to the hypothesis that the resulting conditions at the centrosome led to dynactin obtaining a novel conformation, where $p150^{Glued}$ becomes more flexible, exposing the other regions of the complex. Under these conditions, it is hypothesized that overexpressed p24 results in a "dynamitin-like" dynactin disruption at the centrosome, where the complex is degrading (Quintyne et al., 1999; Quintyne and Schroer, 2002).

In addition to dynactin's role in microtubule anchoring, the interaction of dynein and dynactin allows for correct endomembrane localization (Burkhardt et al., 1997;

Valetti et al., 1999; Vaughan et al., 1999). Although it has been suggested that p24 serves no role in this localization based upon overexpression studies, it was necessary to verify the data by removing p24 expression through knockdown (Figure 6). It was shown that p24 knockdown had no effect on Golgi localization, where it remained next to the nucleus and organized, further supporting previous results that p24 is not required for dynein/dynactin-mediated organization and localization of organelles.

Recent research has manipulated the expression of p24 in order to find its function. When p24 was both overexpressed and knocked down, microtubule organization was lost (Quintyne et al., 1999; McCullough, 2011). Similarly, we observed that p24 knockdown resulted in the loss of microtubule organization (Figure 4), strengthening the hypothesis that p24 is involved in microtubule anchoring.

In addition, previous findings have shown that p24 misexpression effects centrosome integrity, where the number of γ-tubulin foci deviates from the normal 1-2 foci and increases to numbers above this (Quintyne et al., 1999; McCullough 2011). When we knocked down p24, the centrosomes were overduplicated, leading to an increased amount of cells with more than 2 foci, as opposed to our control cells (Figure 5). Although the mechanism through which this abnormality is occurring is unknown, it seems to be correlated with the microtubule disorganization observed when p24 function is lost. These findings further support p24 as a facilitator of microtubule anchoring.

Due to the microtubule disorganization phenotype correlated with p24 misexpression, the state of the dynactin structure at the centrosome in response to p24

misexpression needed to be analyzed. Previous research has shown that the $p150^{Glued}$ subunit, a component of the shoulder/sidearm complex, is found at the centrosome under normal conditions (Quintyne et al., 1999). As a result, staining with antibodies to $p150^{Glued}$ and to Arp1, a component of the minifilament backbone, was optimal for examining the integrity of the dynactin structure. It was found that overexpression of p24 led to the loss of the $p150^{Glued}$ subunit at the centrosome, yet Arp1 remained localized (Quintyne et al., 1999; McCullough, 2011). When p24 was knocked down, both $p150^{Glued}$ and Arp1 remained localized at the centrosome, unaffected (McCullough 2011). These results are interesting, considering the extent of microtubule disorganization that was observed during both overexpression and knockdown of p24. Likewise, when we knocked down p24 expression, the $p150^{Glued}$ subunit remained localized at the centrosome (Figure 7). These findings indicate that as a result of p24 overexpression, the dynactin complex is fragmenting, yet when p24 is knocked down, dynactin remains undamaged, though not fully functional.

Based on the clear increase of perturbed microtubule structures observed in response to p24 misexpression, the next step was to examine any effects on microtubule dynamics. Using microtubule regrowth assays, microtubule dynamics under normal conditions were able to be observed overtime in control cells (Figure 8). However, when the assay was attempted with p24 knockdown cells, the result was inconsistent depolymerization that rendered us unable to make any observations. This may be due to the conditions of the experiment, which required cells to incubate for about four days. The long incubation period may have led to increased extracellular matrix around the

cells that inhibited the nocodazole drug from easily entering the cells, or there was an increase in the stable, non-dynamic population of microtubules due to additional microtubule-actin associations. This observation calls for further modification in the procedure.

The previous findings collectively with our current results allow us to both propose and support a model for p24's function at the centrosome. Under normal conditions, p24 acts as a facilitator for the $p150^{Glued}$ subunit at the centrosome (Figure 9A). This function allows for $p150^{Glued}$ to bind tightly to microtubules and to sustain contact despite the highly tensile forces that full microtubules can place on dynactin molecules at the centrosome (Figure 7A). The force exerted by the microtubule may travel down the $p150^{Glued}$ subunit that is being held rigidly in place by p24, where it is dispersed and shared by the rest of the subunits in the dynactin complex. When p24 is overexpressed, we believe that the excess amount of p24 protein bombards the dynactin complex, displacing the microtubule-bound shoulder/sidearm subdomain from the minifilament backbone. This displacement results in nonfunctional $p150^{Glued}$ subunits that anchor microtubules, which leads to microtubule disorganization (Figure 9B).

On the other hand, when p24 is knocked down, the dynactin complex remains intact at the centrosome. However, with p24 absent, the complex is unstable and the $p150^{Glued}$ subunit is rendered flexible and incapable of anchoring microtubules, resulting in microtubule disorganization once more (Figure 9C).

Although our current results give support to this model, significant research remains to be done to further verify the model. Future directions for this project include

preforming microtubule regrowth assays with p24 both overexpressed and knocked down to detect any aberrations of microtubule regrowth dynamics as a result of the misexpression.

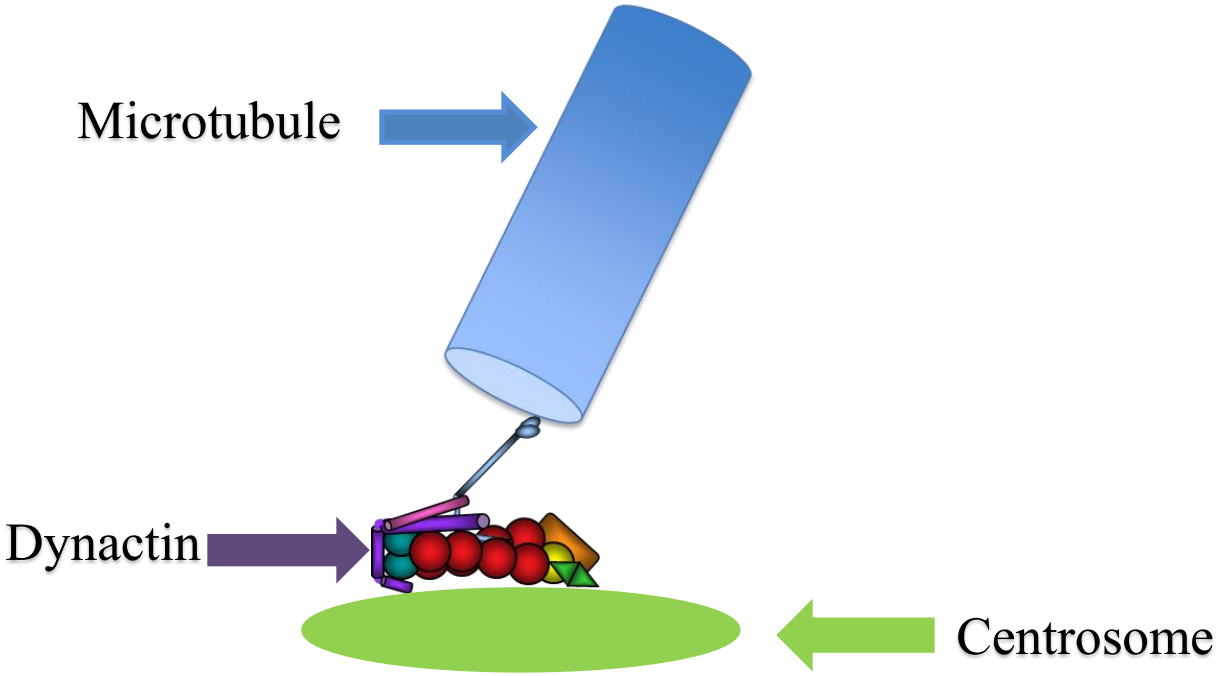


Figure 9A: A model for dynactin under normal conditions: The dynactin complex binds to the microtubule through the $p150^{Glued}$ subunit, which is held rigidly in place by p24. This allows for organized radial arrays of microtubules.

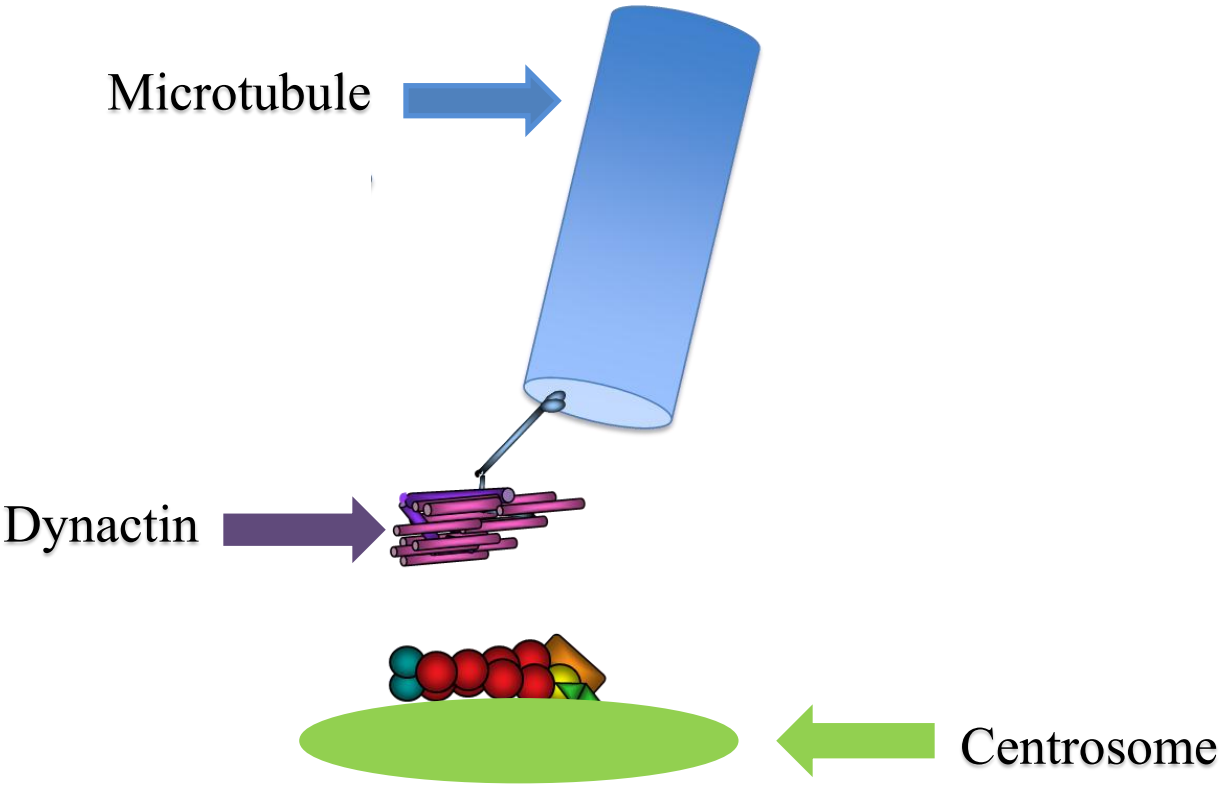


Figure 9B: The overexpression of p24 leads to the bombardment of the complex, thus causing the microtubule-bound shoulder/sidearm complex to dissociate from the minifilament backbone. As a result, microtubules are unable to anchor to the centrosome, leading to microtubule disorganization.

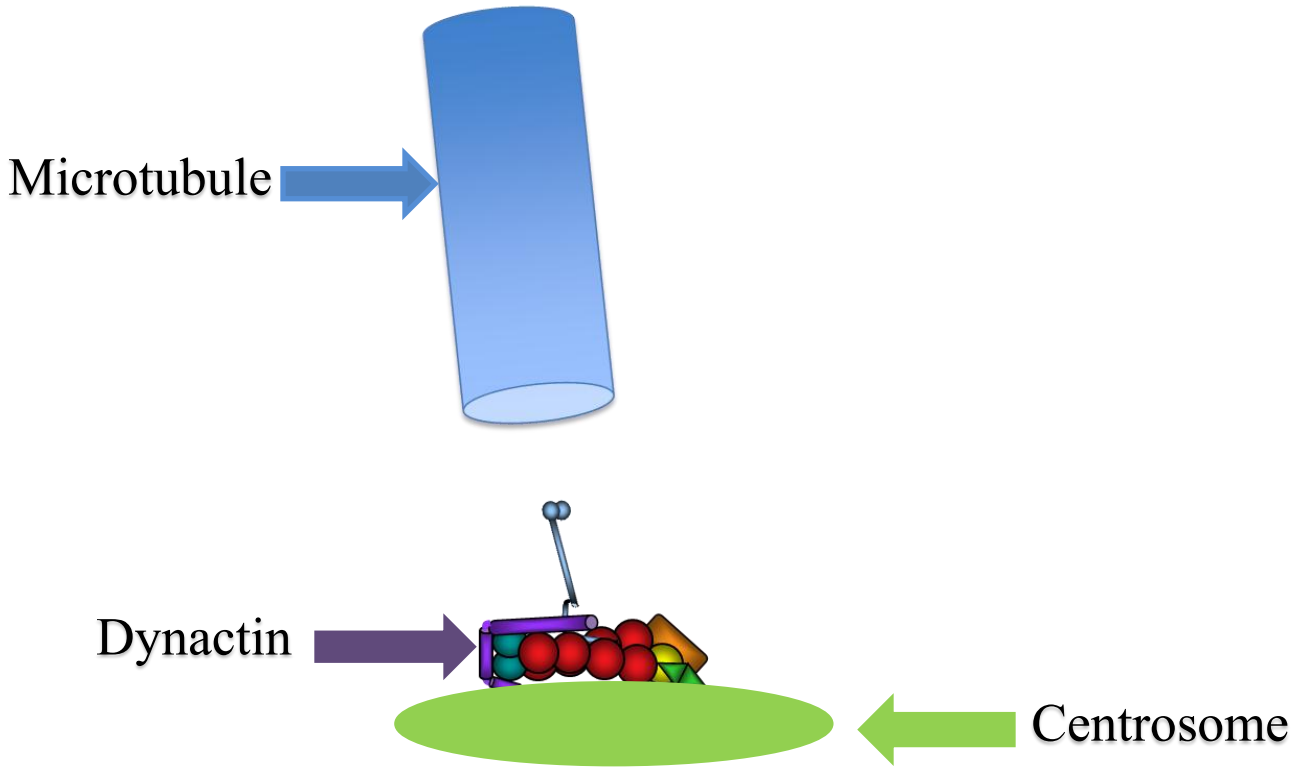


Figure 9C: The knockdown of p24 allows the dynactin complex to remain intact, yet renders the $p150^{Glued}$ subunit flexible. As a result, $p150^{Glued}$ is unable to carry out its function of microtubule binding efficiently, causing microtubules dissociate from the complex.

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