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The Effect of Dynactin Mutations upon Vesicle Trafficking in Living Cells using RNAi

Introduction:

All human cells transport materials from the cell surface to the interior of the cell by forming vesicles under the cell membrane. These vesicles are moved, through endosomes, to the Golgi, and they are sorted and processed along the way. The transport of the vesicles inside the cell is referred to as trafficking. There are two cellular protein complexes that transport vesicles along microtubules during trafficking 1) dynein, which transports materials from the cell surface to the Golgi and 2) kinesin, which moves materials from the Golgi to the periphery of the cell.

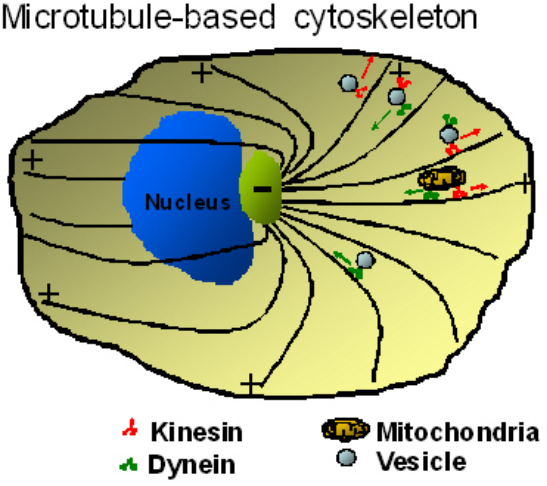

Figure 1: A sketch showing the directionality of dynein and kinesin.

If either of these two "motors" malfunctions, materials cannot be transported correctly and the cell cannot function correctly. Previous work in the lab of Dr. Stephen King at UMKC done in vitro, has shown that the molecule dynactin helps dynein stay on the microtubule tracks,

as dynein moves vesicles inward. In particular, the King lab has shown that the p150 subunit of dynactin plays an essential role in dynein-based trafficking.

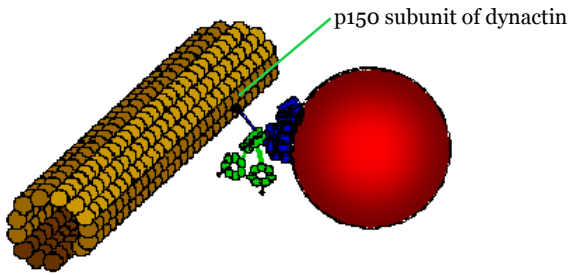

Figure 2: If dynein (shown in green) becomes dissociated from the microtubule, dynactin (in blue) acts as a tether to make dynein more processive. For more detail, see Figure 11 in the Supplemental Images

Based on the *in vitro* experiments there would be a significant decrease in the distance in the cells that have mutant p150, compared to the WT cells.(see Figure 3)

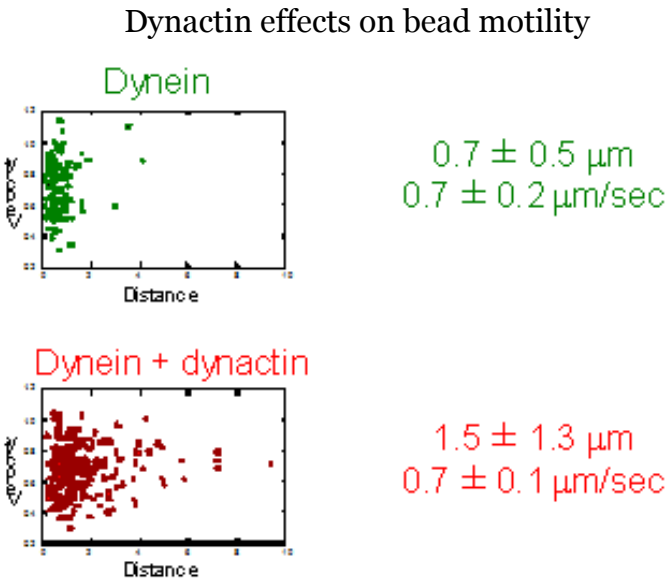

Figure 3: Dot plots showing the affect of p150 mutations *in vitro*.

Recently, it has been shown that a variety of neurodegenerative diseases result from mutations in the p150 subunit of dynactin. This work focuses on mutations of the p150 subunit of dynactin, which we believe to be linked to Amyotrophic Lateral Sclerosis (ALS) and other human motor neuron diseases.

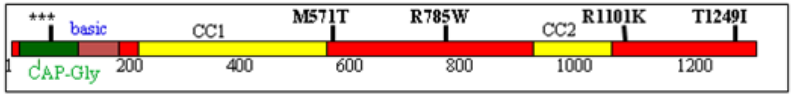

Figure 4: p150 polypeptide sequence The p150 domains discussed in the text include the CAP-Gly and basic microtubule binding domain (MTBDs) and two predicted coiled-coil domains (CC1 and CC2). Amino acid positions of four identified human mutations are in bold, *** show the position of the G59S, G71R, G71E, G71A, T72P, and Q74P mutations.

ALS or "Lou Gehrig's Disease" is a condition that is associated with the degeneration of motor neurons. In humans, the motor neurons are responsible for the transmission of signals along nerve fibers between the brain and muscles. If a trafficking defect occurs in nerve cells, it could be particularly harmful to humans as it may result in the death of the nerve cell and ultimately the complete paralysis of the patient.

For this work, cellular trafficking in live cells with the Wild Type p150 was compared to that of cells with the mutant p150. Distance and velocity of the movements was measured for both dynein and kinesin. The percentage of movements, that is, the number of movements/the total number of vesicles was also calculated. Fluorescence microscopy techniques were used to actually see the trafficking of the fluorescent cargoes and computer analysis software
was used to characterize and quantify the amount of cellular trafficking between the different conditions.

Methods:

Control experiments were conducted primarily to observe dynein/dynactin-based vesicle movements in Wild Type (WT) MRC5 cells. Dr. King's lab has selected these MRC5 cells to study because they are very flat, which makes it easier to observe individual fluorescent vesicles

moving along microtubules inside them. The cells were placed at low temperature (4°C) in protein-free media for five minutes. This low temperature is used to slow the initial step of membrane transport, and the protein-free media will ensure that the cells deplete the external supply of free proteins. This treatment enhances the cells ability to take up our fluorescent marker, which is attached to bovine serum albumin (BSA) in preparation for a 'pulse-chase' style experiment. Texas Red-BODIPY-ceramide (TR-BODIPY) was used as a fluorescent marker. The Texas Red portion is a fluorescent moiety that is attracted to a particular ceramide sphingolipid. TR-BODIPY can bind to any membrane, but has a particularly high affinity for Golgi membranes.

While the cells were still at 4°C, the TR-BODIPY-BSA was added to the media for five minutes of incubation during which the BSA bound to cell surface receptors. The cells were then rinsed twice with protein-free media and then placed into a specialized live cell viewing chamber with complete media, in preparation for a 'pulse-chase' type experiment. The cells were warmed up to 37 °C to allow the TR-BODIPY-BSA to be internalized by endocytosis into endosomes. In the endosomes, the BSA was cleaved from the TR-BODIPY and the TR-BODIPY was transported, in transport vesicles, to the Golgi
. Since the vesicles are spheres of membrane surrounding the material being trafficked, the trafficking of each of the vesicles can be seen, due to the bright fluorescent TR moiety.

The next step of the project was to eliminate the WT p150 and introduce the mutant p150, instead of WT p150. To replace p150 levels in living cells, a siRNA approach was used, in which a plasmid was transfected into the cells (two days before imaging). This plasmid contained genes expressing a Blue Fluorescent Protein (BFP) from a CMV promoter and a small RNA against the 3' UTR region of p150 that targets p150 mRNA for degradation, via standard si knockdown (see Figure 6). Thus the WT p150 was eliminated from the cell and we could identify those cells by their blue color (because of the BFP).

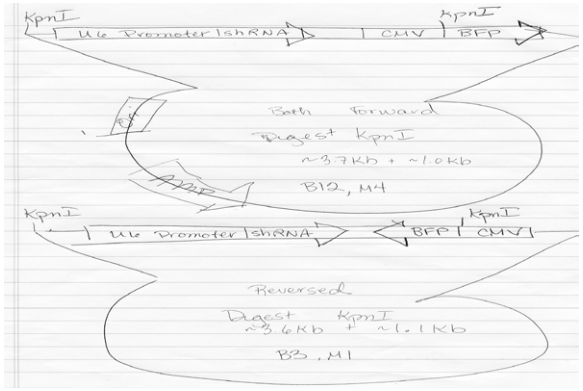

Figure 5: A drawing which shows the plasmid containing the BFP and the RNAi against the untranslated region of p150.

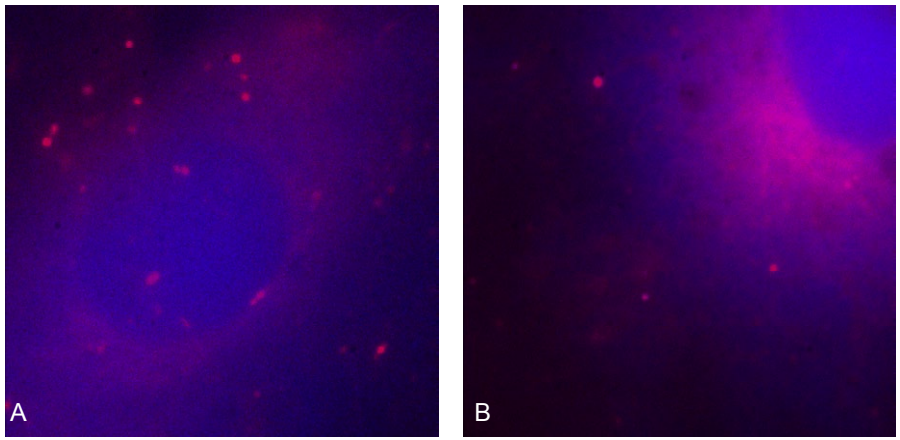

Figure 6: Two cells showing expression of the BFP, but with very different Golgis and percentage of vesicles. (A) at time 28 min., corresponds to video 1, (B) 41 min. corresponds to video 2. The bright spots are the vesicles.

These movements of the fluorescent vesicles were recorded on the departmental High Resolution Microscope in 50-second movies of 500 frames each. In WT cells, the trafficking was robust for up to 60 minutes. These movies were then analyzed using MetaMorph imaging software to determine the frequency of motility events and the distance and velocity of each individual movement. The movements can be seen as Figure 7

demonstrates. The program will help track the vesicles and produce a Kymograph (Figure 8). The Kymograph is a graph of the intensity of the fluorescence along the line drawn on the movie. The top of the graph corresponds to frame 0, time = 0 seconds, and the bottom is frame 500, time = 50 seconds. Thus, the distance and slope of the graph will give us an indication of what trafficking occurs in the cells. Once the kymograph (Figure 8) has been made, the actual distance in microns and the velocity in microns/sec. can be calculated (see Figure 9). These calculations take into account the number of pixels that correspond to one micron from previous calibrations of the microscope with a micrometer.

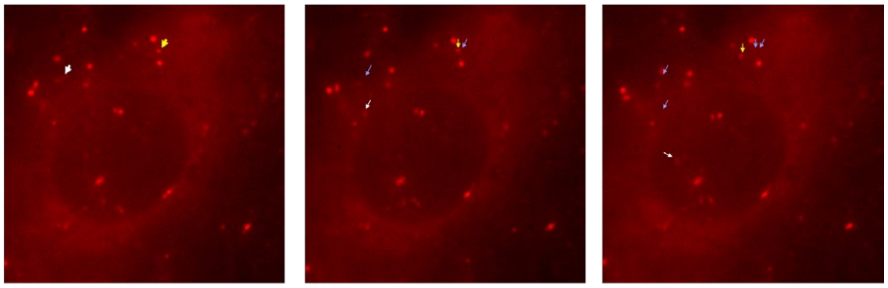

Figure 7: The arrows indicate the position of the vesicles at the times indicated. The blue arrows indicate the vesicle's position in the previous images. A line drawn between these points is used to determine the area graphed. The computer tracks the movements in this area and the Kymograph is produced, as is shown in the screen snapshot show in Figure 3.

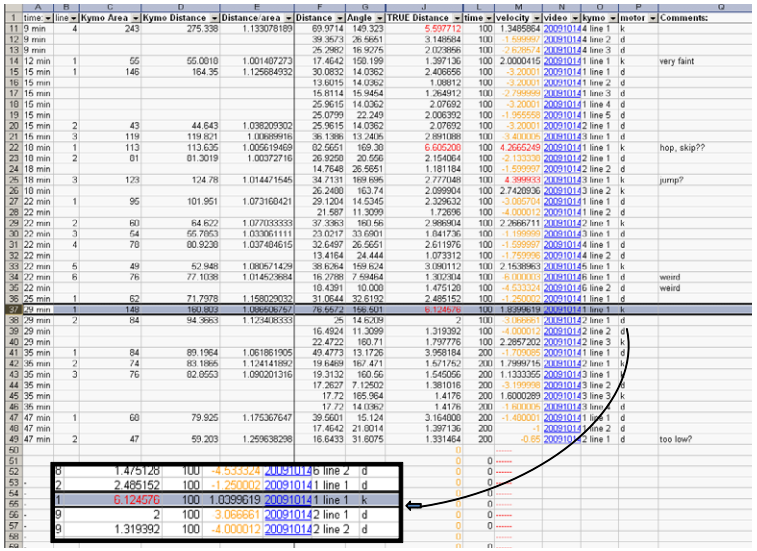

Figure 9 (Bottom, Left): From the distance and angle of the line on the kymograph, we can account for the calibration of the microscope and calculate the distance and angle moved.

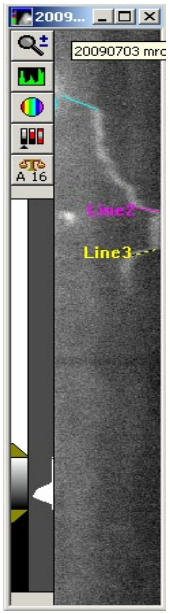

Figure 8: The angle of these lines is used to determine the velocity at which the vesicles moved and the length is used to determine the distance moved.

Results:

After the cells were transfected with the plasmid containing the RNAi and the BFP, I compared distance and velocity of dynein and kinesin-based movements as well as the percentages for each. A summary of this analysis can be seen in Table 1. This shows that while the distance and velocity remained comparable, the percentage of movements was significantly altered. An example of this decrease in percentage of movements can be seen in the supporting.

Cargo Manipulation	GFP-Rab6	BOPDIPY-Ceramide	BODIPY-Ceramide UTR2-BFP
% cells with moves	100	100	48
% moving vesicles	96	64	8
Kinesin Velocity	1.9 +/- 0.7	2.7 +/- 1.2*	2.0 +/- 0.9*
Kinesin distance	2.7 +/- 1.6	3.0 +/- 1.9	2.4 +/- 1.3
n	313	314	403
Dynein velocity	2.1 +/- 0.9	2.7 +/- 1.2	2.1 +/- 1.0
Dynein distance	2.5 +/- 1.4	2.8 +/- 1.6	2.3 +/- 1.1
n	176	315	448

Table 1: A comparison of the distance and velocity for Dynein and Kinesin and the percentage of movements in each experiment. * = *P*< 001 Student's t-test

While the velocity of kinesin in the BFP-UTR2 cells was shown to be significantly different between two cell populations, the distance for kinesin and both the velocity and distance of dynein were quite similar. This indicates that while there is a visual difference between cells that have the RNAi and WT cells, it is not the velocity and distance of the movements that is changed. Thus we looked at the percentages of movements, that is, how many of the vesicles in a cell actually move. In the cells with the BFP-UTR2, there is a drastic reduction in the percentage of movements when compared to WT cells.

What was most conspicuous was the fact that some cells had the plasmid in them, as seen by the expression of BFP, but behaved like WT cells. We thus concluded that in the blue cells, some were displaying the mutant phenotype, while some were displaying a WT phenotype.We concluded that the RNAi targeting the untranslated region of p150 was not being consistently effective. The plasmid was clearly in the cells, but the RNAi did not seem to be causing a phenotype, even though it is on the same plasmid. We expect the RNAi was made, but the level may not have been high enough to remove all cellular p150 RNAs.

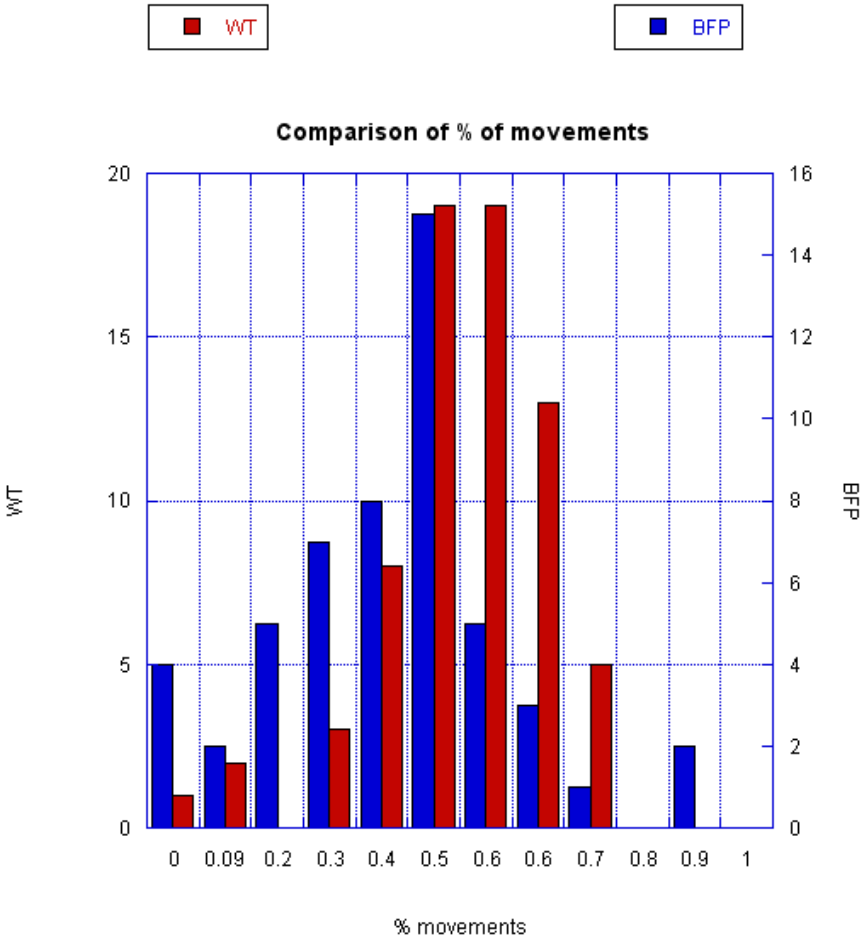

Figure10: Graph showing the difference in percentage of movements between WT and BFP-UTR2 cells. The WT percentages are shown in red and are on the left y-axis. The BFP percentages are shown in blue and are on the right y-axis.

Conclusions:

The RNAi used did indeed affect the global function of dynein-based transport. However, the distance and velocity of individual movements, when they occurred, were not affected. The affect of the RNAi was a decrease in the number of movements. This indicates that *when* a vesicle moves in a mutant cell, it moves similarly to a vesicle in a WT cell. Also, the number of vesicles was decreased in the mutant cells. Since p150 is only known to be involved in the binding of vesicles to microtubules, it is not immediately apparent if a mutation in p150 would affect vesicle formation or only its transport. Likewise, the decrease in the number of kinesin-based movements as a result of the p150 mutation indicates that any mutation in the cell can affect processes other than that with which it is directly related.

An RNAi to a different region of p150, such as in the coding region, may have different results. Experiments using the same techniques with a new RNAi may have a more consistent effect on p150. If mutations in the coding region of p150 have consistent similar results to the blue cells with a mutant phenotype seen in these experiments, it may indicate that this is the best approach to take in future studies. This can also be examined by introducing the RNAi into live nerve cells and seeing if they cause similar affects to those seen in ALS patients. If this is the case, RNAi may also be a means of reversing ALS by eliminating the mutant p150 and replacing it with the WT p150.

Supplemental Images:

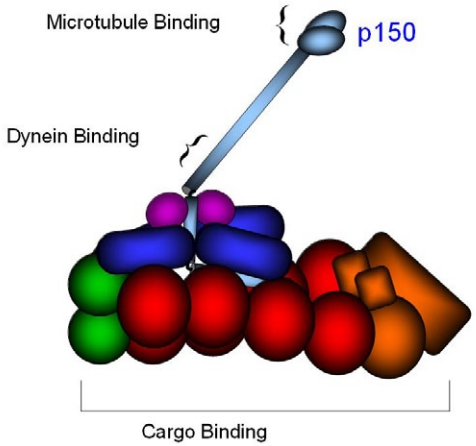

Figure 11: A representation of the structure of dynactin. The microtubule binding domain is what mutations in p150 are affecting.

Notes

1. A microtubule-binding domain in dynactin increases dynein processivity by skating along microtubules.
Culver-Hanlon TL, Lex SA, Stephens AD, Quintyne NJ, King SJ.
Nat Cell Biol. 2006 Mar;8(3):264-70. Epub 2006 Feb 12.PMID: 16474384

2. Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein.
Gill SR, Schroer TA, Szilak I, Steuer ER, Sheetz MP, Cleveland DW.
J Cell Biol. 1991 Dec;115(6):1639-50.PMID: 1836789

3. MetaMorph version 6.1. Universal Imaging Corp.

4. Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein.
Gill SR, Schroer TA, Szilak I, Steuer ER, Sheetz MP, Cleveland DW.
J Cell Biol. 1991 Dec;115(6):1639-50.PMID: 1836789

5. Molecular Probes, Inc. NBD- and BODIPY Dye-Labeled Sphingolipids (2003)

6. Constructed by Dr. Margaret Kincaid