

Potential Role of Acetylated-Tubulin in Sympathetic Nerves of Chick Embryos

Daniel Kvistad
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I. Introduction

Acetylated Tubulin is known to play a role in microtubule based protein trafficking (Gardiner, J., Barton, D., Marc, J., & Overall, R. 2007). Acetylation is a unique post translational modification which occurs on lysine 40 of alpha-tubulin. The enzyme which carries out the acetylation/deacetylation of this site is still unknown. The mechanism by which this modification could influence microtubule functions also remains unclear. Although much is not discovered yet, research has shown that acetylation of alpha tubulin on lysine 40 is common and can be found on stable microtubules in most cell types (Hammond, J. W., Cai, D., & Verhey, K. J., 2008). While acetylation has been discovered to not be essential for organismal survival, recent work shows that it plays a positive role in motor-based protein trafficking in mammals (Hammond, J. W., et al., 2008). Recent work has also highlighted the role of hyperacetylation of microtubules in neuronal cells. The research has shown that hyperacetylation caused kinesin-1- transport (motor protein trafficking) to be redirected to neuronal projections, or neurites and enhanced the transport of BDNF vesicles (Hammond, J. W., et al., 2008). BDNF vesicles contain brain-derived neurotrophic factor, which is in the family of neuron growth factors. BDNF is known to support the survival of existing neurons, and promote the development and differentiation of young neurons and synapses (Acheson A., Conover J. C., Fandl J. P., DeChiara T. M., Russell M., Thadani A., Squinto S. P., Yancopoulos G. D., Lindsay R.M., 1995).

Since acetylated tubulin may play a role in promoting microtubule based protein trafficking, including BDNF, it may be logical to hypothesize that differentiating neurons will have a greater presence of acetylated alpha-tubulin. Acetylated Alpha-tubulin may be more conducive to the trafficking of growth factors and proteins essential for development than deacetylated alpha-tubulin.

The model system used in this experiment was a ten day old chick embryo of the *Gallus gallus*. Their nervous systems are not highly developed by the tenth day, thus preventing them from feeling pain (Hamberger, V & Levi-Montalcini, R. 1949). The sympathetic nerve chains and dorsal root ganglia were dissected from the embryo's spinal column. This specimen was chosen for our experiment because their nervous system had the same mechanisms and processes of human nerves, thus what was studied can most likely be translatable to human neurons and glia (Miura, K., Himeno, S., Koide, N., & Imura, N., 2000).

By studying microtubule acetylation in glial and neuronal cells, we can have a better understand the role they may play in differentiation and proliferation of glial and neuronal cells.

The hypothesis tested was that as glial and neuronal cells differentiate, acetylated alpha-tubulin is present. In order to test this hypothesis we used the technique of immunofluorescent staining. We cultured neurons and glia from the ten day old chick embryo and targeted the alpha tubulin and acetylated tubulin as our two main antigens of interest, staining DNA for reference. Different stages of differentiation were examined as well. Neurons and glia are known to grow in a radial manner, with new growth migrating beyond their predecessors, thus forming layers in an inward-outward manner, meaning the youngest growth is furthest from the central point of growth (Nadarajah, B., Parnavelas, J., 2002). We hope that further research can be done with this topic because the function of acetylated tubulin is largely unclear (Aguilar, A., Becker, L., Tedeschi, T., Heller, S., Iomini, C., & Nachury, M. V. 2014).

II. Materials and Methods

The experimental procedure began with the dissection of the 10 day old chick embryos. The materials involved and the procedures were carried out as documented by Professor Morris for the Primary Culture Of Chick Embryonic Peripheral Neurons 1 (2014a).

The next step was to determine whether there was growth in our cultures. This procedure and the materials involved can be found in Primary Culture Of Chick Embryonic Peripheral Neurons 2 (2014b).

After confirming neuronal and glial growth, we fixed the cultures in MeOH or Formaldehyde/glutaraldehyde. The fixation was followed by immunofluorescent labeling. The procedures for these steps is as documented in Immunofluor staining of SU embryos - MeOH fixation (2013). The recipe for the formaldehyde/glutaraldehyde fix can be found in Immunofluorescence of chick neuronal primary cultures (RLM - 10/15/2014).

In our three experimental conditions, we labeled with DM1A to target alpha-tubulin and fluoresce green. We used anti-acetylated-tubulin (Sigma T-6793-0.2m) to label acetylated tubulin, and the secondary, GAM tetramethyl rhodamine which is fluorescent red. We also used a Hoechst label to fluoresce DNA blue.

We used two different concentrations of the anti-acetylated tubulin antibody, 1:1000, and 1:500. One MeOH and one formaldehyde fixation received the 1:1000 concentration, and these were referred to as experimental 1s. Experimental 2 was a MeOH fix with a 1:500 concentration of the anti acetylated tubulin antibody.

Data analysis

Our data was collected using imageJ to measure the distance of a neuron or glial cell from its point of growth, which in all our samples was a larger clump of cell bodies from which neurons and glia grew outward. The distance is a measure of cell age, seeing as growth is directed outward, new growth will be further from the central point, while older growth will be closer.

We also measured the brightness of tetramethyl rhodamine antibodies bound to anti-acetylated tubulin antibodies using imageJ. We measured brightness by using a ratio of the brightness of a traced region on either an axon or glial cell to the brightness of the background around it. This helped to eliminate noise from brighter regions nearby our points of interest, thereby isolating the brightness of our points of interest without noise.

III. Results

The two experimental conditions which were successfully cultured, stained and mounted were experimental 1 with a MeOH fixation, and experimental 1 with the Formaldehyde/glutaraldehyde fixation. Both experimental 1s had an anti-acetylated-tubulin antibody concentrations of 1:1000.

Experimental 2, which had a higher antibody concentration (1:500 vs 1:1000) and was a MeOH fixation did not have any growth.

The analyzed brightness ratios of neurons and glia and their distances from the central growth point were recorded from both experimental 1s. Four recordings were made per image, one young and one old for both neurons and a glia. An example of the recordings can be seen in Figure 1. The results within each sample are visualized in Figure 6.

The results from these recordings are visualized in the following figures.

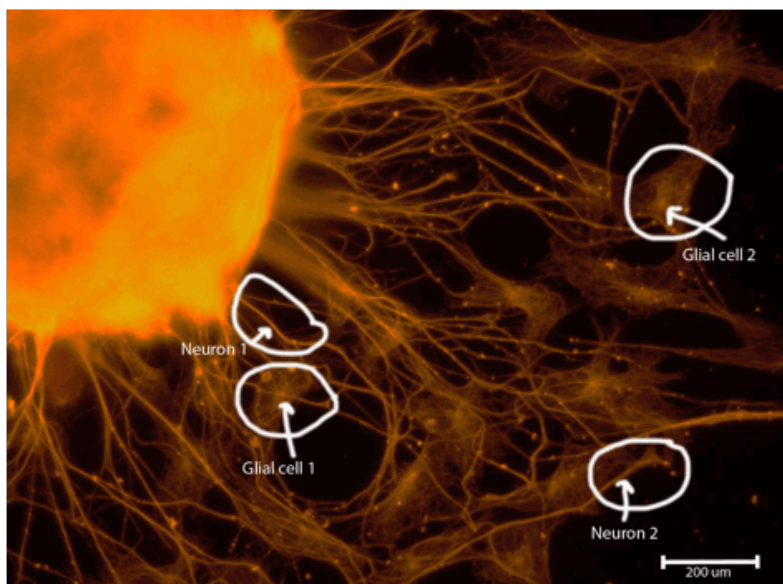
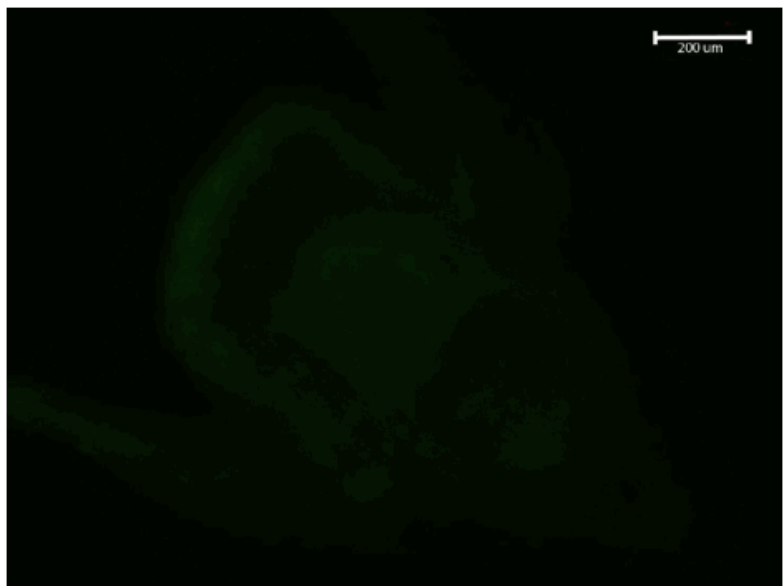


Figure 1. This image is an example of where the recordings were made for our young and old neurons/glia. Neuron/glia 1 are “old” and neuron/glia 2 are “young”.



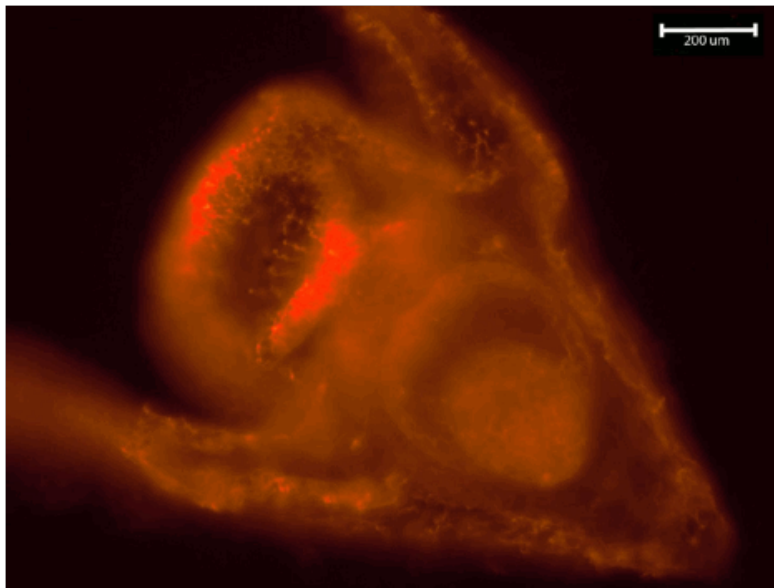


Figure 2. The left image shows the DM1A staining of alpha-tubulin in the positive control with sea urchin embryos. The right image shows the glowing tetramethyl rhodamine secondary antibody which is targeting anti-acetylated tubulin.

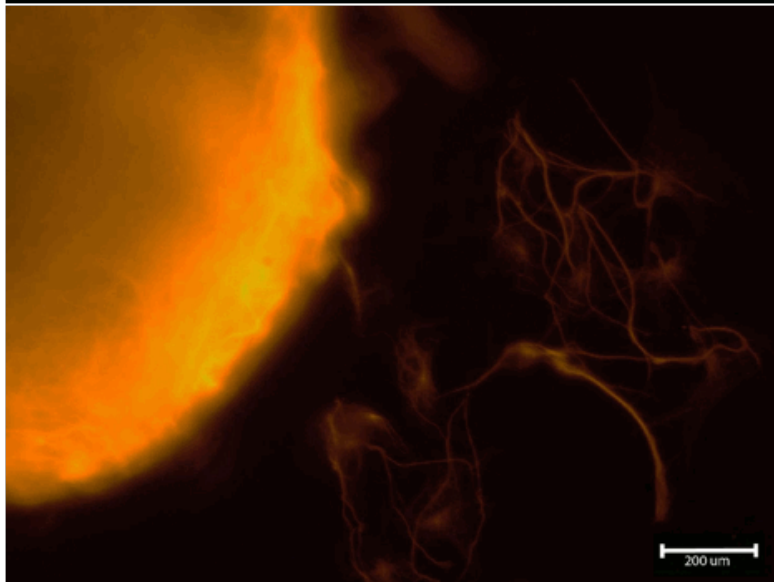
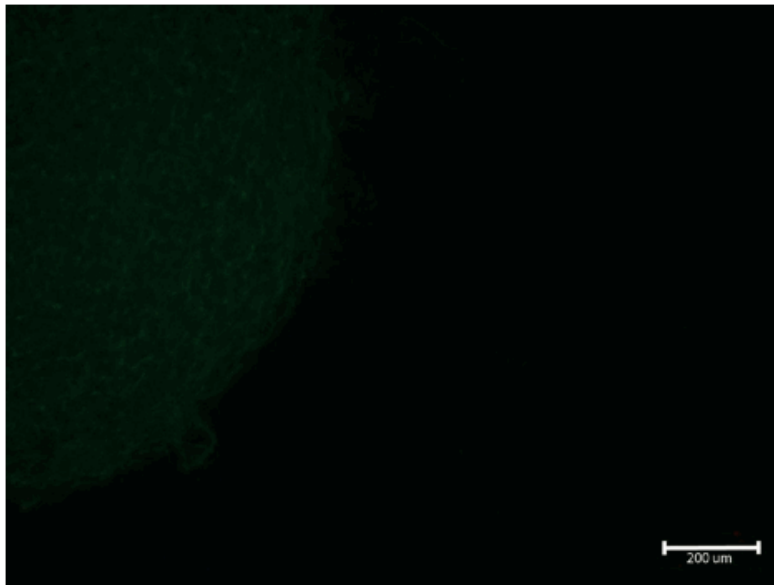


Figure 3. Both images are of neurons and glia in the negative control formaldehyde fixation. Both images show the background noise present in our samples. Image exposure time was 1.5 seconds.

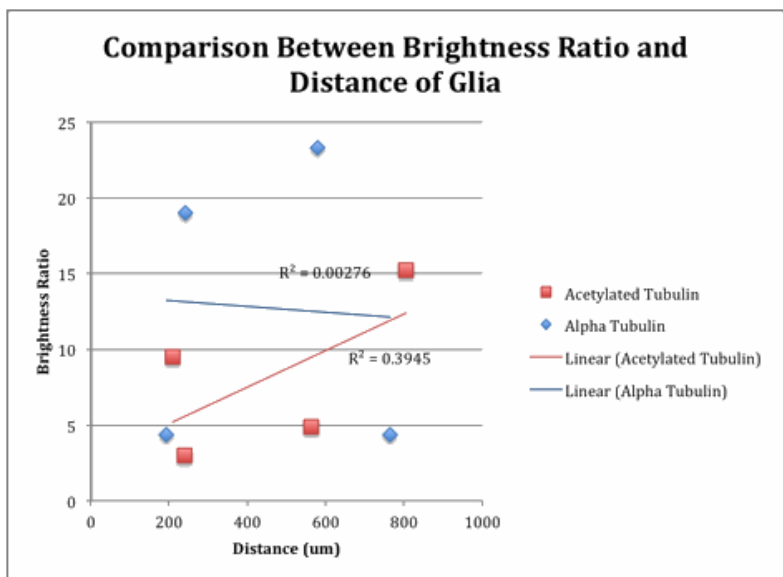


Figure 4. This graph has a comparison of brightness ratio, on the y axis, to distance (age) on the x axis for cultured glial cells. Brightness ratio is a representation of the ratio between the brightness of the target cell and its adjacent background. This is a combination of data from both experimental 1s which showed results. The larger the distance, the younger the cell; the greater the brightness ratio, the higher the presence of the antigen. Antigen type is labeled in the key.

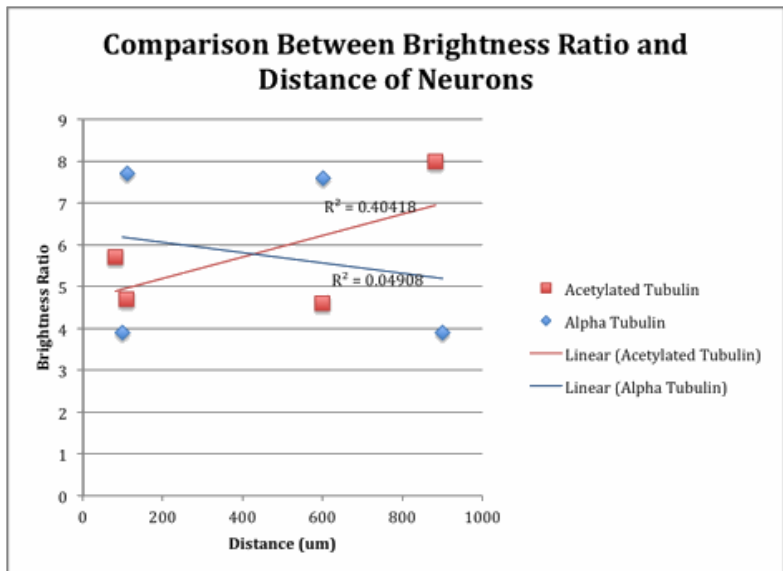


Figure 5. This graph compares brightness ratio on the y axis to distance (age) on the x axis for neurons in our samples. Brightness ratio is a representation of the ratio between the brightness of the target cell and its adjacent background. The larger the distance, the younger the cell, and greater the brightness ratio, the higher the presence of the antigen. Antigen type is labeled in the key.

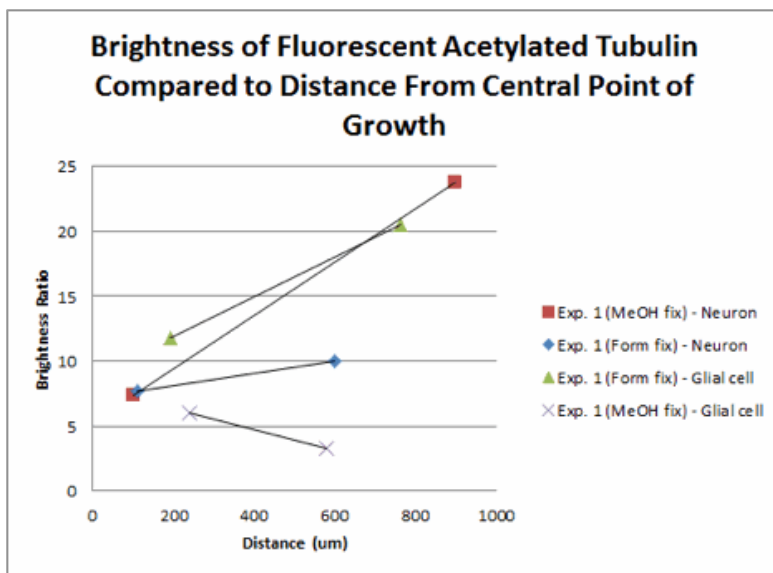


Figure 6. This graph shows the difference in brightness ratio of the fluorescent acetylated tubulin in neurons and glia within each sample. Brightness ratio is a representation of the ratio between the brightness of the target cell and its adjacent background. On each sample, the brightness ratio of one close and one far glial cell or neuron were recorded.

The results from the positive control showed that both the anti-alpha tubulin DM1A, and the anti-acetylated plus the secondary, tetramethyl rhodamine, stained their antigens. The results can be seen in Figure 2.

The results from the negative control in the MeOH fixation show that there was no growth in the culture, which means, most likely, that an error was made in the procedure. The results from the negative control in Formaldehyde/glutaraldehyde fixation show that there was some auto fluorescence in the samples. This is evident by the fact that there was glowing in both red (anti-acetylated) and green (anti-alpha tubulin) as seen in Figure 3. This glow was taken into account when analyzing the experimentals, it was assumed that the glow present in the negative control was present in all cultures as background noise.

IV. Discussion:

The hypothesis that as glial and neuronal cells differentiate, acetylated alpha-tubulin is present was not supported by our data. The data as seen in figures 4 and 5 show that there was no clear trend of increasing acetylated tubulin in younger neurons and glia. However, the youngest neurons and glia both showed an increase in the brightness ratio of acetylated tubulin, with a decrease in alpha tubulin. These results are intriguing, because they could show that acetylated tubulin is present in high quantities during a specific, earlier stage of differentiation. These results would need further exploration in order to see if this is an actual trend or if is just the case in the limited samples that were tested.

Figure 6 shows how the brightness ratio of neurons and glia increased as distance increased for each condition. The positive trend of increased brightness with distance was present in both pairs of neurons studied, but only present in one pair of glial cells. This data is pointing in the direction of supporting our hypothesis, but cannot be considered statistically significant due to the small sample size.

If these trends were to continue with a much greater number of samples and measurements, there could be evidence to support our hypothesis. The results could be explained by suggesting that the youngest neurons and glia are in a state of hyperacetylation of their microtubules. This state of hyperacetylation is shown to increase motor protein trafficking to neurites and the transport of BDNF vesicles (Hammond, J. W., et al., 2008). The transport of BDNF to neurites could be promoting differentiation of the most distal ends of neurons and glia as research shows (Acheson A, et al., 1995).

We were unable to collect a large amount of data, due to the limitations of the class, as well as the lack of growth in some of our cultures.

If the experiment was repeated a thousand times and received the same results for all those times, it could be concluded that our hypothesis was supported. The hypothesis was that in differentiating neurons and glia, acetylated tubulin is present. The data would support just that, showing a spike in the presence of acetylated tubulin in the youngest growth, followed by a decline as they age.

There were many sources of error in our experiment. One major source of error is that there may have been mistakes made with adding something to a culture which did not belong, such as an antibody solution. Another source of error could be exposing our samples to too much light, which would cause bleaching.

Since there was some autofluorescence in our negative control, it was expected that the same level of brightness was present as background noise in our experimentals and positive control. Because of this, it was hard to be sure whether the fluorescence in our experimentals and positive controls was actual antigen bound antibodies fluorescing, or just noise.

To refine this experiment I would do more controls, as one of the negative and one of the positive controls showed no growth. These controls are essential to know if our antibodies were working as we hoped. I would also redo everything with less light involved in order to prevent bleaching, which may have influenced how our samples came out.

V. Sources:

- Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (March 1995). "A BDNF autocrine loop in adult sensory neurons prevents cell death". *Nature* **374** (6521): 450–3.
- Aguilar, A., Becker, L., Tedeschi, T., Heller, S., Iomini, C., & Nachury, M. V. (2014). α -Tubulin K40 Acetylation is Required for Contact Inhibition of Proliferation and Cell-Substrate Adhesion. *Molecular biology of the cell*, mbc-E13.

- Gardiner, J., Barton, D., Marc, J., & Overall, R. (2007). Potential Role of Tubulin Acetylation and Microtubule Based Protein Trafficking in Familial Dysautonomia. *Traffic*, 8(9), 1145-1149.
- Hamberger, V & Levi-Montalcini, R. (1949) Proliferation, differentiation and degeneration in the spinal ganglion of the chick embryo under normal and experimental condition. *J Exp Zool* 111, 457-502.
- Hammond, J. W., Cai, D., & Verhey, K. J. (2008). Tubulin modifications and their cellular functions. *Current opinion in cell biology*, 20(1), 71-76.
- Miura, K., Himeno, S., Koide, N., & Imura, N. (2000). Effects of methylmercury and inorganic mercury on the growth of nerve fibers in cultured chick dorsal root ganglia. *The Tohoku journal of experimental medicine*, 192(3), 195-210.
- Morris, Robert L. (2014a) Primary Culture Of Chick Embryonic Peripheral Neurons 1: dissection. Retrieved from http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_1_Dissection_2014.htm
- Morris, Robert L. (2014b) Primary Culture Of Chick Embryonic Peripheral Neurons 2: Observation of Live Unlabeled Cells. Retrieved from http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_2_ObserveLiveCells_2014.htm
- Morris, Robert L. (2013) Immunofluor staining of SU embryos – MeOH Fix. Retrieved from http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_4_Immunofluor_staining_of_SU_for_use_in_Neurobio.htm
- Nadarajah B, Parnavelas J (2002). "Modes of neuronal migration in the developing cerebral cortex". *Nature Reviews Neuroscience* 3 (6): 423-32. doi:10.1038/nrn845. PMID 12042877.
- Pearson, J., Dancis, K., Axelrod, F., & Grover, N. (1975). The sural nerve in familial dysautonomia. *Journal of Neuropathology & Experimental Neurology*, 34(5), 413-424.
- Reed, N. A., Cai, D., Blasius, T. L., Jih, G. T., Meyhofer, E., Gaertig, J., & Verhey, K. J. (2006). Microtubule acetylation promotes kinesin-1 binding and transport. *Current biology*, 16(21), 2166-2172.
- Vale, R. D. (2003). The molecular motor toolbox for intracellular transport. *Cell*, 112(4), 467-480.