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An Investigation into the Relationship Between the Activation of AMP Kinase and the Acetylation of Microtubules

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Abstract:

Microtubules, or cytoskeletal polymers composed of the protein tubulin, form long hollow tubes in the cell and are responsible for many critical roles. Previous research has shown that depletion of ATP causes microtubules to become stable, i.e. resistant to depolymerization. It has also been shown that enhanced stability of microtubules correlates with increased tubulin acetylation, a common microtubule posttranslation modification. ATP-depletion is a severe metabolic stressor, and as such, we expect that this treatment would activate AMP kinase, an enzyme deemed the "master regulator" of metabolism. When activated by a variety of stressors, this enzyme can initiate a program of limited 'self-destruction' called autophagy, which requires microtubules for the transport of autophagosomes. By using western blot analysis, I ascertained that tubulin acetylation increases upon ATP-depletion and that AMP kinase is activated upon ATP depletion in mouse embryo fibroblasts (C3H 10T1/2). By western blot analysis and immunofluorescence microscopy I also confirmed that ATP-depletion causes the acetylation of microtubules and that these acetylated microtubules are resistant to depolymerization by nocodazole. These results suggest a link between tubulin acetylation and the control of ATP levels in the cells.

Introduction:

Many cellular functions rely on the microtubule cytoskeletal system, including guiding intracellular transport, anchoring membrane-enclosed organelles, forming the mitotic spindle during mitosis, and also serving as the bundle core of cilia and flagella. Microtubules are polymers composed of the protein tubulin. The tubulin protein is a dimer made from one α-subunit and one β-subunit. They polymerize into proto-filaments, which then form stiff hollow microtubules in the cytoplasm of the cell (Alberts et al., 2010).

Microtubules exhibit a behavior called dynamic instability, in which individual microtubules can depolymerize abruptly, remain stable, or polymerize (Alberts et al., 2010). These behaviors seem to occur randomly and the reason behind this behavior is not well understood. Dynamic microtubules are short-lived, only remaining for less than a minute to up to 5-10 minutes. At the same time in a cell there are also stable microtubules that remain (neither polymerizing nor depolymerizing) for more than one hour (Webster et al., 1987). One possible explanation for the dynamic instability that is being heavily investigated is that posttranslational modifications (PTMs) to tubulin are responsible for differentially stabilizing subsets of microtubules.

PTMs are being investigated as a possible source of microtubule stability. Most PTMs occur on the alpha subunits of tubulin and they are reversible. Gundersen and colleagues discovered PTMs to tubulin in 1987. They found that post-polymerization, the C- terminal tyrosine amino acid was removed from the α-tubulin in a subset of microtubules (Gundersen et al., 1987). Since then, additional tubulin posttranslational modifications have been identified, and not only include detyrosination, but also

acetylation, polyglutamylation, glutamylation, glycylation, phosphorylation and palmitoylation. (Verhey & Gaertig., 2007; Janke & Bulinski., 2011). While many PTMs occur on the C-terminal, some PTMs can be found jutting out from the lattice structure of the microtubule.

The exact function and purpose of PTMs are not well understood, but there is a significant amount of evidence that PTMs are important for interactions between microtubules and microtubule-associated proteins (MAPS) (Peris et al., 2009). In the case of detyrosination it has been shown that a microtubule-depolymerizing motor is inhibited by the detyrosination of tubulin (Peris et al., 2009). Many studies have shown a correlation between tubulin PTMs and stability or longevity of microtubules (reviewed in Wloga & Gaertig, 2010).

Webster et al. (1987) were the first to note that there was a difference in turnover between tyrosinated and detyrosinated microtubules. That is, a decrease in the dynamics of microtubules correlates with increased levels of detyrosination. However, it is still not clear whether detyrosination leads to the stability of the microtubules or if stable microtubules are conducive to detyrosination, though there is some evidence that the later is true (Khawaja et al., 1988). One explanation offered to explain why some microtubules are stable while adjacent microtubules remain dynamic, is that there is a capping mechanism on microtubules that is responsible for stabilizing them and then making microtubules more conducive to detyrosination (Infante et al., 2000).

Histone deacetylase 6 (HDAC6) was identified as the deacetylase that specifically removes the acetyl group on tubulin (Hubbert et al., 2002). Currently, there are many investigations of HDAC6 as it relates to cancer (Aldana-Masangkay and Sakamoto,

2010). For example, the stabilization of microtubules leads to increased levels of HDAC6-mediated deacetylation of alpha tubulin. Over expression of HDAC6 leads to a decrease in tubulin acetylation. Thus, HDAC6 plays a role in the dynamics of microtubules and ultimately in cellular functions such as mitosis and motility. Additionally, the existence of a deacetylase for the acetyl groups that have been added to microtubules shows that acetylation is a reversible process, but still the function of the acetylation is unknown.

Following the discovery of tubulin detyrosinated, tubulin acetylation was found to occur on Lysine 40 of the α-subunit of tubulin in the lumen of the microtubule. In a study in human fibroblast cells Webster et al. (1989) found that acetylated microtubules had a slower turnover. They specifically chose the human fibroblast cell line, because their microtubules tend to have few detyrosinated tubulin, a decision that allowed them to more accurately investigate the dynamics between acetylation and stable microtubules. Some of their results even suggested that acetylation is independent of the stability of the microtubules. Certain treatments or conditions will alter the dynamic instability of microtubules, causing the majority of microtubules either to depolymerize or to become very stable (Alberts et al., 2010). For example, drugs such as nocodazole and colchicine cause widespread depolymerization, while drugs like taxol stabilize microtubules so they are no longer as dynamic (Alberts et al., 2010).

In 1981, Bershadsky and Gelfand showed that by depleting mouse fibroblast cells of ATP, there was a decrease in the depolymerization of microtubules (Bershadsky and Gelfand, 1981). That is, the microtubules become less dynamic and more stable. They even resisted depolymerization by colchicine. Gundersen et al (1987) showed that

microtubules made stable by ATP depletion were heavily detyrosinated. Although the method of ATP-depletion is not physiological, cells can encounter sudden stresses that lead to drops in ATP concentrations, such as hypoxia, glucose deficiency, and decreased pH levels. All of these discoveries hinted that there is a connection between the control of metabolism (as represented by the depletion of ATP) and microtubules. Furthermore, there might be a link between cellular energy and posttranslational modifications of microtubules, but more experimentation is necessary.

In a recent review, Cassimeris et al. (2012) discussed evidence for the close association between acetylated microtubules and the mitochondria, which alludes to the idea that microtubules play a role in metabolism. It is possible that dynamic instability is part of the metabolic function of the microtubules (Cassimeris et al., 2012). In addition to being closely associated with the mitochondria, acetylated microtubules have been shown to be crucial for endoplasmic reticulum (ER) dynamics (Friedman et al., 2010). After being treated with Trichostatin A, an HDAC6 inhibitor they saw the hyperacetylation of microtubules coupled by an increase in the ER dynamics.

Metabolic stressors can induce a form of cell protection/termination called autophagy. This process involves the formation of vesicles, which fuse with lysosomes, which then move along microtubules via motor proteins to return essential building blocks to the cell. Autophagy is an important form of degradation in eukaryotic cells. In a study of starvation-induced autophagy, in all microtubules there was an increase in tubulin acetylation between basal and starvation conditions (Geeraert et al., 2009). Over the past decade much evidence, regarding the relationship of HDAC6 to autophagy has accumulated (Matthias et al., 2008; Xie et al., 2010). Microtubule dynamics are also

critical for autophagy because they recruit markers of autophagosome formation. Geeraert et al. (2010) found that hyperacetylation of labile and stable microtubules was a characteristic of cells that were nutrient deprived, a finding that suggests that HDAC6 is being inhibited. HDAC6 has been deemed a new cellular stress surveillance factor because of how it responds to cytotoxic accumulation of protein aggregates (Matthias et al., 2008).

Cells have evolved a protective mechanism to handle stresses caused by starvation, hypoxia (loss of oxygen), and other conditions that lead to a drop in ATP levels. One of these protective mechanisms starts with the activation of AMP-activated protein kinase (AMPK), a heterotrimeric enzyme with an α-subunit that is anabolic, and β and γ subunits that are regulatory. A drop in ATP levels is matched by an increase in AMP. Increased levels of AMP are responsible for allosterically promoting the phosphorylation of the α-subunit of AMP kinase by LKB1 (Woods et al., 2003). Likewise, a decrease in AMP means less phosphorylation of AMP kinase through LKB1. LKB1 is one of several AMPK kinases and it accounts for most of AMPK activity in a purified rat liver (Wood et al., 2003).

AMPK is the regulator of many downstream targets. TSC2, a mediator between cellular energy response, and cell growth and survival, is phosphorylated by AMPK. Activation of TSC2 can be stimulated by treatment with 2-Deoxyglucose, an ATP-depleting drug (Inoki et al., 2003). One downstream target that is of particular interested is called Mammalian target of rapamycin complex 1 (mTORC1) (Weinberg, 2007). mTORC1 is important for regulating cell growth and autophagy (Gwinn, D.M. et al.,

2008; Milhaylova et al., 2011), and this signaling pathway is being explored as a potential target for new anticancer therapies (Weinberg, 2007).

AMP kinase is currently being investigated in the context of metabolic syndrome and diabetes (Zhang et al., 2009). Once activated, AMP kinase shuts down specific activities in the cell that consume ATP, and stimulates other activities to increase energy stores. These include the stimulation of both fatty acid oxidation and glucose uptake in various muscle tissues (Minokoshi et al., 2002; Merrill et al., 1997). In 1999, AMPK activation by AICA-riboside treatment was tied to the translocation of the major glucose transporter, GLUT4, in skeletal muscle (Kurth-Kraczek et al., 1999). When GLUT4 is at the plasma membrane surface of a cell it increases glucose uptake. These results suggest that an increased activation of AMPK could be a useful treatment for type II diabetes. These findings also strongly suggest that AMPK serves important metabolic roles in the cell.

Recently, Nakano et al. (2010) showed that a microtubule binding protein, CLIP-170, is phosphorylated by AMPK. CLIP-170 controls microtubule dynamics at the plus end. In 293T, human kidney epithelial cells, the inhibition of AMPK slowed tubulin polymerization (Nakano et al, 2010). Paclitaxel, a drug that prevents microtubules from depolymerizing, and nocodazole, a microtubule-depolymerizing reagent, caused abnormally sized focal adhesions, which regulate the effects of the extracellular matrix adhesion on the cell. The change in size is regulated by microtubule dynamics that are controlled by AMPK-dependent phosphorylation of CLIP-170. Evidence from this study establishes a link between the activation of AMPK and microtubule dynamics.

In this study, I first determined whether the depletion of ATP causes the acetylation of microtubules in a 10 T ½ cell line by utilizing immunofluorescence and immunoblotting. Using the same methods I tested if acetylated microtubules are more resistance to a depolymerizing reagent (nocodazole) than were non-acetylated microtubules. Next I wanted to explore a possible relationship between the activation of AMPK and tubulin acetylation. To do this I compared the amount of ATP-depleted cells with control cells, using western blot analysis. The results suggest that AMPK activation might stimulate microtubule acetylation.

Methods:

Cell Culture

10 T ½ cells were grown in a 5% carbon air humidified incubator at 37°C in MEM supplemented with 10% FBS, sodium pyruvate, penicillin-streptomycil, and glutamine. For preparation of immunofluorescence, the cells were grown on glass cover slips 1-2 days before the start of the experiment. During sub-culturing, trypsin-ETDA was used to remove cells from the dishes.

Immunofluorescence (Indirect)

10 T ½ cells were grown on glass cover slips, and treated with a combination of 10mM 2-deoxyglucose, 20mM sodium azide for 2 hours, or 5 μg/ml nocodazole for one hour, with both 2-deoxyglucose and sodium azide followed by a nocodazole treatment, or were left untreated. Cells were fixed after the treatments by placing the cover slips in ice-cold (4° C) methanol for ten minutes. The cover slips were incubated with an antibody directed against anti-acetylated tubulin 1:500 in calcium and magnesium free phosphate buffered saline, CMF PBS, for one hour in a moist chamber at 37 ° C. After the primary antibody incubation, and washing in CMF-PBS, the cover slips were then incubated with Alexa Red Goat anti-mouse secondary antibody 1:300 in CMF PBS for one hour in a moist chamber at 37 ° C. A third incubation was completed with FITC –conjugated anti-alpha-tubulin antibody 1:150 in CMF PBS for 1 hour. After the third incubation the cover slips were mounted on slides using a Prolong Gold anti-fade reagent with DAPI, and viewed on a fluorescence microscope.

Western Blotting (Immunoblotting)

Cultures of cells were treated as described above and cell lysates were made by Professor Danowski. 20 μl of samples were loaded into the SDS-page gel. Molecular weight markers were loaded as 8 μl aliquots. Wet transfer was used in order to guarantee the best transfer of proteins to the nitrocellulose membrane. The Transfer buffer was 75ml of 10X Running Buffer, 1ml of 10% SDS, and 20% of MeOH to 1 liter of milliQ water. The blots were then placed in 5% nonfat dry milk blocking solution for one hour at room temperature. In one experiment the blot was incubated in 1:5000 dilution of α-acetylated tubulin and 1:5000 dilution of GAPDH in 5% nonfat dry milk in TTBS (1XTTBS and 0.1% Tween) over night at 4°C, and in another the same antibody was used but the incubation was only 1 hour at room temperature. For the secondary antibody we used a 1:5000 dilution of Goat α-Mouse-Horseadish Peroxidase diluted in TTBS. After washes in TTBS, Goat α-Mouse in TTBS was used as the secondary antibody.

Another immunoblot was performed. This time the four treatments were loaded into a 4-15% precast gel twice with the Precision Plus molecular weight markers. A wet transfer for 70 minutes at 70 volts was used to transfer the proteins from the gel to the nitrocellulose membrane. After the transfer the blot was placed in blocking solution (5% nonfat dry milk in TTBS) and then the blot was washed. One half was incubated overnight in 4° C in 1:2500 anti-phosphoAMPK Beta in Bovine Serum Albumin (BSA) and other half with 1:2500 dilution of total AMPK Beta and 1:2500 dilution of anti-tubulin DMIA in BSA. The next day there were three ten-minute washes in TTBS before the secondary antibodies. The secondary antibodies for the anti-phosphoAMPK Beta was 1:5000 dilution of Goat anti-Rabbit in TTBS and for the total AMPK Beta was 1:5000

dilution of Goat anti-Rabbit and Goat anti-Mouse in TTBS. After 1-hour of incubation in secondary antibodies the membranes had three-10 minute washes in TTBS.

After all the blots were incubated in appropriate antibodies and washed properly the final step is immunodetection. The chemiluminescence reagent was the Thermo Scientific Pierce ECL Plus Substrate for Western blotting detection kit. Images of the membranes were taken using the Fiji LAS-3000 imager.

Results:

Immunofluorescence

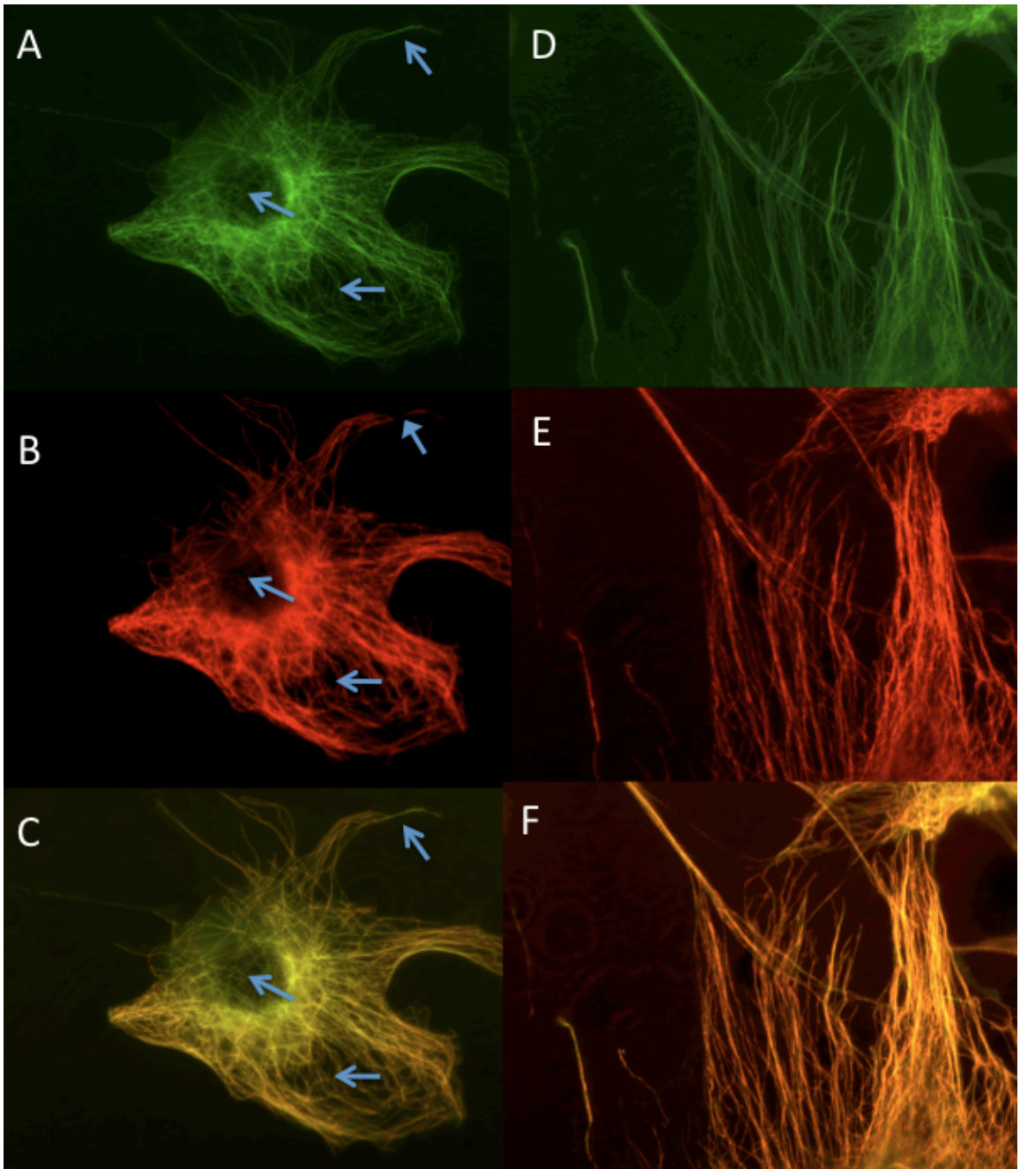

Figure 1 Comparison of control (A-C) and ATP-depleted (D-F) C3H10T1/2 cells in a 120μm image. Total tubulin is shown in green (A&D); acetylated tubulin is shown in red (B&E). Overlays of total tubulin and acetylated tubulin is shown in C & F.

In order to determine whether ATP-depletion caused an increase in tubulin acetylation, immunofluorescence microscopy was performed on C3H10T1/2 mouse fibroblasts. Control cells contained a large number of acetylated microtubules. The acetylation was not continuous on the microtubules. Rather, it occurred in discreet patches. This is seen in Figure 1 (a, b, c). When cells were treated with 10 mM 2-deoxygcose and 20mM Na-azide for 2 hours, I saw a number of changes. The experiment was repeated four times. On two occasions, I saw a large amount of detached cells that were treated with ATP-depleting drugs. Still, the cells that remained attached to the cover slips had microtubules that were highly acetylated (Fig. 1, c, d, e). Not only was the acetylation pattern of the ATP depleted cells more continuous along the length of the microtubule than it is in the untreated cells, but it was also more robust. That is, the acetylation pattern seems to be in thicker patches in the cells that were depleted of ATP.

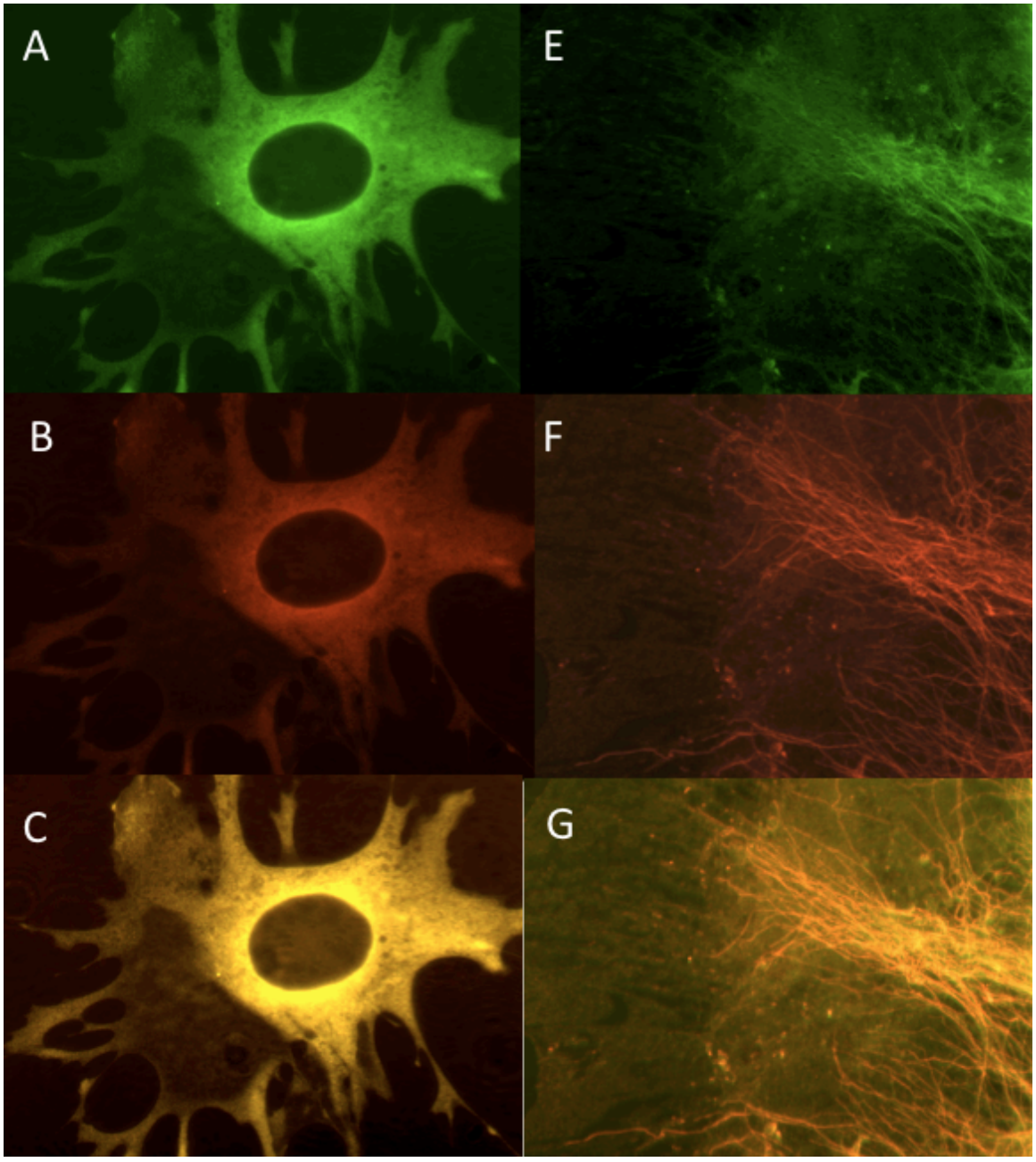

Figure 2. Immunofluorescence of a 10 T ½ cell that was treated with nocodazole for 1 hour (A-C) in a 120 μm image. A) Green fluorescence is total tubulin. B) Red fluorescence is acetylated tubulin. C) Overlay of acetylated tubulin and total tubulin. Immunofluorescence of the side of a 10 T ½ cell after two hours of ATP depletion followed by a 1-hour treatment with nocodazole (E-G). E) Green fluorescence is total tubulin. F) Red fluorescence is acetylated tubulin. G) Overlay of acetylated tubulin and total tubulin.

Since it has been reported by others that ATP-depletion stabilizes microtubules to the effects of microtubule depolymerizing agents (Bershadsky and Gelfand, 1981), I next

wanted to determine the extent to which microtubules in ATP-depleting medium are able to resist depolymerization induced by 5 uM nocodazole. This experiment was performed 5 times, and in most cases, the cells treated with ATP-depleting drugs and nocodazole rounded up and detached from the cover slips. Nevertheless, I was able to find a few cells that remained attached. An example is shown in Figure 2. In the other 3 experiments it was difficult to find cells that had not detached during treatment, but when I did, they appeared similar to the cell seen in Figure 2, d, e, and f. As expected, nocodazole induces the depolymerization of the majority of the microtubules in the cell (Figure 2, a, b, c). In Figure 2 c, we can see that the tubulin, though not in polymer form, is still acetylated. Figure 2 D-F shows only a portion of a large cell. In the overlay (Figure2 f) it is clear that acetylation did lead to stabilized microtubules, as all the acetylated microtubules remained after treatment with nocodazole. Since there were significant amount of cells that detached, this experiment should be repeated to verify the results presented here.

Immunoblotting

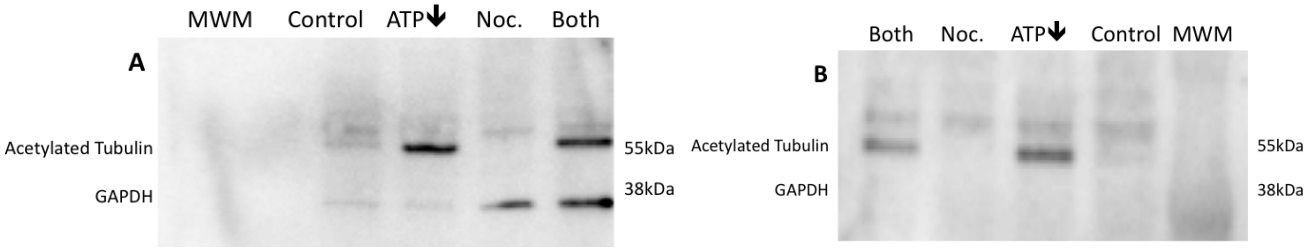

Figure 3. A western blot reveals that treatment with Sodium Azide and 2-Deoxyglucose to deplete the cell of ATP leads to an increase in acetylated tubulin. A) A 90 second exposure shows that cells depleted of ATP (ATPê) had elevated amounts of Acetylated tubulin. Cells depleted of ATP and also treated with nocodazole (Both) also had an increase in the amount of acetylated tubulin. Cells just treated with nocodazole (Noc.) did not have elevated amounts of acetylated tubulin. B) A 60 second exposure shows that cells depleted of ATP (ATPê) had elevated amounts of Acetylated tubulin. Cells depleted of ATP and treated with nocodazole (Both) also had an increase in the amount of acetylated tubulin. Cells just treated with nocodazole (Noc.) did not have elevated amounts of acetylated tubulin. In both experiments, GAPDH was used as a loading control. The complete transfer of GAPDH was not successful.

Immunoblotting was performed to quantify the amount of acetylated tubulin in control cell lysates, ATP depleted cell lysates, nocodazole treated cell lysates, and ATP depleted and nocodazole treated cell lysates. Figure 3 shows the results of 2 separate western blotting experiments. In both experiments, we can see bands at 55 kDa, which represent acetylated tubulin. Both experiments show that there is a significant difference in the amount of acetylated tubulin between the control cells and the ATP depleted cells. This is clear when comparing the intensity of the 55 kD bands in control and ATP-depleted cell lysates, that is the ATP-depleted lysates are more intense; therefore, they have more acetylated tubulin. Similarly to the immunofluorescence experiments, I also wanted to confirm that acetylation leads to more stable microtubules; therefore, the same necessary treatments were also run on the western blot. In both blots, for the lanes containing lysates made from nocodazole treated cells, I saw no bands at 55 kD. In blot A, there is a faint band that is running higher that 55 kD, and is present in the other lanes as well, so it's likely to be nonspecific. I would therefore say that little to no acetylated tubulin was seen in lysates made from cells treated with nocodazole.

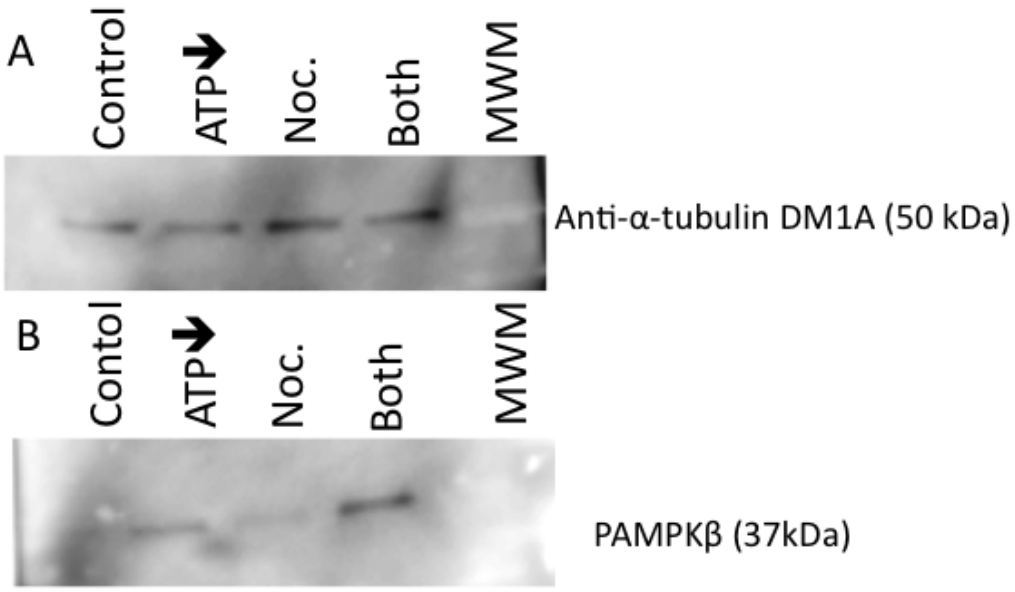

Figure4. Western blots that were run on the same gel with the same lysates, but in different wells. A) The results of a western blot for total α-tubulin (50kDa). Treatments are untreated cells (Control), cells treated with Sodium Azide and 2-Deoxyglucose (ATPê), cells treated with nocodazole (Noc.), and cells treated with Sodium Azide and 2-Deoxyglucose, and nocodazole (Both). In all treatments the bans are or equal thickness. B) The results of a western blot for Phospho-AMPKinaseβeta (PAMPKβ) (37kDa). Treatments are untreated cells (Control), cells treated with Sodium Azide and 2-Deoxyglucose (ATPê), cells treated with nocodazole (Noc.), and cells treated with Sodium Azide and 2-Deoxyglucose, and nocodazole (Both). The bans vary in intensity.

Immunoblotting was also used to quantify how much phospho-AMPKβ is present in cell lysates that were made from cells that were either untreated, treated with ATP-depleting drugs, treated with nocodazole, or treated with ATP-depleting drugs and nocodazole. Control cells do not have a band at 37 kDa period. However, the ATP-depleted cells have a low intensity band at 37 kDa, which is indicative of some phospho-AMPKβ. The nocodazole treated cells show a small amount of phospho-AMPKβ. The most intense band is for the cells that were treated with ATP-depleting drugs followed by a nocodazole treatment. The α-tubulin is the loading control and the bands were of comparable intensity and size in all four lanes. This can be seen in Figure4A.

Discussion:

ATP Depletion Causes the Acetylation of Microtubules in 10T1/2 Fibroblast Cells

My results show that when 10T1/2 cells are depleted of ATP, the microtubules become more heavily acetylated. Gundersen and colleagues (1987) showed that increased tubulin detyrosination was stimulated in TC-7, human polarized intestinal cells, when depleted of ATP. So, it was not too surprising to find that tubulin acetylation also increased.

Since drug-induced ATP-depletion mimics a metabolic stress that is physiologically possible in the cell, this finding suggests that acetylated microtubules might somehow be linked to the cellular metabolic stress response. During starvation-induced autophagy, microtubules show an increase in tubulin acetylation between basal and starvation conditions (Geeraert et al., 2009). My results support this previous finding, because in both cases, a metabolic stress causes an increase in the amount of tubulin acetylation.

It is possible that the increase in acetylated tubulin functions to enhance microtubule-based vesicle transport, allowing the cell to distribute resources when energy is deprived. Maruta et al. (1987) have speculated that the acetyl groups added to tubulin are important for the binding of various microtubule-associated proteins (MAPS). Along the microtubules, there are many kinesins and dyneins, which can transport vesicles into and away from the cell. During a time of cellular stress, there is likely to be a shift in mobility of microtubule-dependent vesicle transport, such that the cell can conserve energy and survive the stress. Yet, kinesin and dynein utilize a significant amount of ATP in order to move along the microtubules; therefore, these additional acetyl groups on the

microtubules could be functioning to prevent the movement of kinesin and dynein, in order to preserve energy in the cell.

Whether in the control cells or in the cells depleted of ATP, there were subsets of microtubules that were acetylated and subsets that were not acetylated. It is not well understood whether these different subsets of microtubules play different roles in the cell. What is better understood are some of the tubulin acetyltransferases, that include ARD1-NAT1 (arrest-defective 1-amino-terminal, α-amino, acetyltransferase 1), ELP complex (elongator protein complex), MEC17/αTAT1 (α-tubulin N-acetyltransferase), and GCN5 (general control of amino acid synthesis 5) (Janke and Bulinski, 2011). MEC-17, which is related to GCN-5, it acetylates Lysine 40 only. When MEC-17 is knocked out it leads to less stable microtubules (Akella et al., 2010). Whether all of these acetyltransferases are highly conserved is a matter that needs to be explored.

Since my results are only accounting for the Lysine 40 acetylation site of the α-tubulin it is important to realize that different sites of acetylation might follow different patterns and therefore not become acetylated when the cell is depleted of ATP. Again adding to the complexity of the function of acetylation.

Recent evidence shows that acetylation is not a process that is limited to the α-subunit. The β-subunit of the tubulin is also a location for acetylation (Chu et al, 2011). San, an acetyltransferase is responsible for the acetylation of the β-tubulin at Lysine 252 in 293T cells (Chu et al., 2011). Moreover, the depletion of San actually increased microtubules regrowth rate *in vivo,* a finding that suggests that an acetylation on the β-subunit can negatively regulate the polymerization of tubulin into the microtubules. In San-siRNA-treated cells, the same researchers found that there were no differences in the

amount of acetylation at the lysine 40 site on the alpha tubulin in cells with San-siRNA and cells without San-siRNA. This result suggests that perhaps different acetyltransferases exist, which can acetylate different lysine on both the α and β subunits of tubulin. Having a better understanding of where acetylation can happen on the microtubules will afford more insight into the function of this posttranslational modification. Another possible aspect to further explore is why the researchers found that after 80% San depletion in HeLa cells there were no microtubule defects, but there was a significant increase in the microtubule regrowth.

Acetylated Microtubules are More Resistant to Depolymerization by Nocodazole Reagent than the Non-Acetylated Microtubules

My results from immunofluorescence microscopy suggest that the acetylated microtubules were more stable than were the non-acetylated microtubules. When treated with nocodazole, a microtubule depolymerizing reagent, acetylated microtubules remained polymerized in the cell and other microtubules depolymerized.

What these results do not address is whether or not microtubule stabilization is a cause or an effect of acetylation. In support of the idea that microtubules are stabilized before they are acetylated, research in *Chlamydomonas reinhardtii*, a single celled green alga, clearly showed that in axonemes and basal bodies, microtubules are usually stabilized by cross-linking structures (LeDizet and Piperno, 1986). Therefore, it is possible that the cytoplasmic microtubules that are resistant to depolymerizing reagents are already stabilized by other proteins or even by being closely associated with other microtubules (LeDizet and Piperno, 1986). It is possible that acetylation only assists the

binding of other cellular proteins to cause stabilization. For example, Microtubule-Associated Proteins (MAPS) are more likely to cause stability by binding to acetylated tubulin (Maruta et al., 1987). Some MAPS are being deemed microtubule destabilizers, such as katanin and spastin (Janke and Bulinski, 2011). Perhaps these proteins specifically bind to the posttranslationally modified microtubules to cause the stabilizing or destabilizing effect.

Having acetylated microtubules (i.e. more stable ones) is important for vesicle transport needed for autophagy. Again the acetylated microtubules might be playing an important role for MAPS, which normally utilize a lot of ATP while moving along the length of the microtubules. My results do not clarify if stabilization is a cause or an effect of acetylation and in regards to other PTMs there is evidence going both ways.

Another distinction that should be investigated is what is the difference between acetylated microtubules that are stable in the cell and non-acetylated microtubules that are stable in the same cell. It was already mentioned that stability seems to take two forms that is a long-term (about 1-hour) and a short-term (about 5-10 minutes). ATP-depletion seems to make the majority of microtubules resistant to depolymerization, which is different from the long-term and short-term stability and even different from taxol-induced stability. It is possible that the stability that is caused by ATP-depletion has a specific function that is important for autophagy.

ATP Depletion Leads to an Increase in Phospho-AMPKβ in 10T1/2 Cells

ATP-depletion caused an increase in phospho-AMPKβ, indicating that AMPK had been activated. Moreover, even more phospho-AMPKβ was present in the cells that

were treated with ATP-depleting drugs and than with nocodazole. Though it is already very well established that AMPK has an important role in controlling the metabolic rate in the cell, these results suggest the possibility that the activation of AMPK might be upstream of the acetylases that are responsible for the acetylation of microtubules.

Apparently, phosphorylation of the α-subunit is required for the activation of AMPK (Hawley et al., 1996). But the β-subunit is also phosphorylated and recently, it has been shown to be important for AMPK activation, as well (Oakhill et al, 2010; Sanders et al, 2007). The β-subunit contains two domains that are highly conserved in eukaryotes. The ASC domain is required for the complex formation of the entire αβγ complex (Hardie et al, 2003). The other domain is called KIS and while it interacts with other kinase subunits it also is responsible for binding glycogen, ultimately allowing AMPK to act as a glycogen sensor. In order to prove definitely that ATP-depletion leads to the activation of AMPK, one needs to also determine whether the α-subunit is phosphorylated on Threonine 172 (Hawley et al, 1996).

Recently, it was shown that the myristoylation of the β-subunit plays a critical role in this phosphorylation process (Oakhill et al, 2010). That is, without the myristoylation of the β-subunit, the upstream kinases, which phosphorylate AMPK, would not function. If there is a direct relationship between myristoylation and phosphorylation of AMPKβ, then it is possible that my results are a good indicator of the overall activity of AMPK, but evidence of this does not exist. What these results could mean is that because there is more phospho-AMPKβ that the β-subunit has more opportunity to be myristoylated by the upstream factors (again if phosphorylation is directly related).

Some research suggests that there is an autophosphorylation that occurs at the serine 108 of AMPKβ (Sanders et al., 2007). This autophosphorylation site happens to also be in the glycogen-binding domain (GBD) of the β-subunit. Moreover, researchers found that an AMPK, which lacks the GBD of the β-subunit, did not change the ability of AMP to allosterically activate AMPK (Sanders et al, 2007). A-769662, a small molecule activator of AMPK was no longer able to allosterically affect AMPK in an AMPK complex that lacked the GBD.

Clearly AMPK does not directly acetylate microtubules, as structurally AMPK would not be able to fit into the lumen. Understanding if acetylation is truly limited to tubulin when they are in microtubule form is one aspect of the current understanding that this research brings into question. I was surprised to see that α-tubulin, after being depolymerized were still acetylated in all of the immunofluorescence experiments. Immunoblotting experiments confirmed that some tubulin were acetylated post-depolymerization, a confirmation that suggests that acetylation and deacetylation do not only occur on intact microtubules.

Mutational analysis on one of the β-subunit isoforms, demonstrated that an S108A mutation, or when the serine in position 108 was changed to an alanine to prevent phosphorylation of the β-subunit, reduced AMPK activity by 60% (Warden et al, 2001). This indicates that AMPKβ phosphorylation is important for the overall activity of AMPK. This finding supports my hypothesis that there is a relationship as part of the response of the cell to metabolic stress, between AMPK activation and the acetylation of microtubules.

Recently, there has been a lot of research pertaining to the relationship of AMPK and the relationship of acetylated tubulin to autophagy. Autophagy is a form of cell death in which the lysosomes are responsible for the digestion of the cell's cytoplasmic material. The stimulation of kinesin-1/JNK pathway is necessary for the initiation of autophagy. Geeraert et al. (2010) showed that kinesin-I requires hyperacetylated microtubules. Their experiments focused on tubulin hyperacetylation caused by nutrients deprivation, so presumably, AMPK has been activated.

As more downstream targets of AMPK are being discovered, more functions of AMPK are also being found. One function that is currently under investigation is the role of AMPK in autophagy. For example, there is a lot of evidence that shows that AMPK phosphorylates ULK1, a critical component to autophagy (Mihaylova and Shaw, 2011). Though no direct connections between AMPK and tubulin acetylation have been discovered, my research suggests that there is a link that is related to the cellular metabolic stress response. When understood in the context of other research, it suggests that perhaps this relationship is critical for the cell to carry out autophagy.

Looking Forward

It is possible and plausible that there is more than one protein responsible for stabilizing microtubules, and that acetylated microtubules are an effect of this stabilization, not a cause (LeDizet and Piperno, 1986). My results support the idea that there is a subset of stable microtubules that are acetylated, but it still needs to be confirmed if stability is a cause, effect, or unrelated to acetylation. A closer look at the acetylation patterns when cells are being depleted of ATP could offer further insight.

Already different locations for acetylation and different acetylases are being discovered (Chu et al, 2011) a fact, which foreshadows the complexity of this posttranslational modification. Still when considering these results and the results of others it is arguable that AMPK activation is an upstream of the acetylase of microtubules. To make this research more conclusive it would be beneficial to inhibit AMPK and see if there is still acetylation of microtubules. Experiments can be done to determine this. For example, siRNA knockout can inhibit the tubulin acetylases. Also, if a particular MAP is knocked-out and then AMPK is activated, but microtubules do not become acetylated, it is possible that we can understand the downstream protein that likely exists between AMPK and an acetylase. Additionally, one can chemically inhibit AMPK and determine whether this alters the levels of acetylated microtubules in cells.

In the 10 T ½ cell line it is possible that ATP depletion is not the most effective way of creating a stressful environment, as complete depletion of ATP is physiologically rare. The method of ATP depletion seems to be effective, but during two separate immunofluorescence experiments, the cells that were treated with Sodium Azide and 2-Deoxyglucose rounded up and detached. It is necessary to have both of these drugs in order to block both oxidative and glycolytic pathways. It is possible that the 10 T ½ cells were already under stress during incubation, or perhaps from nutrient deficient medium, ultimately making the cells incapable of coping with such an intense cellular stress.

A lot of concern about the use of ATP depletion as an effective way to manipulate cells is necessary. For example, one group of researchers hypothesized that microtubule-targeting drugs would inhibit the hypoxia-inducible factor-1 (HIF-1) (Escuin et al, 2005). What they found is that both microtubule-stabilizing and microtubule-destabilizing drugs

inhibit HIF-1, a change that does not allow HIF-1 to inhibit tumor growth and tumor angiogenesis.

My results suggest that acetylation happens in a similar pattern as detyrosination (Gundersen et al, 1987). That is, instead of the deacetylase acting solely on the microtubules they also act on the tubulin subunits. The acetylated tubulin subunits remained in the cytoplasm. I assume, although it has not been proven, that the HDAC6 deacetylase acts on these subunits. Then when microtubules are needed in the cytoplasm an unmodified tubulin is ready to polymerize. Some research shows that αTAT (alpha-tubulin acetyltransferase), is an enzyme that acetylates tubulin dimers as well as microtubules (Maruta et al, 1986). Further exploration into when a tubulin becomes acetylated and deacetylated is another inlet into defining the purpose of acetylation.

There are many possibilities for research that will increase our understanding of the relationship between the AMPK activation and the acetylation of microtubules. Understanding more about the regulation of acetylation will bring us closer to understanding it's function and the function of AMPK. If acetylation is related to the stability of microtubules this knowledge can be used to pursue pharmacological options for people with cancer, type II diabetes, and metabolic syndrome.

Bibliography:

Akella, J. S., D. Wloga, J. Kim, N. G. Starotina, S. Lyon-Abbott, N. S. Morrissette, S. T. Dougan, E. T. Kipreos, and J. Gaertig. "MEC-17 Is an Alpha-tubulin Acetyltransferase." *Nature* 467.7312 (2010): 218-U111.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. *Essential Cell Biology*. New York, NY: Garland Science Pub., 2010.

Aldana-Masangkay, G. I., and Sakamoto K. M. "The Role of HDAC6 in Cancer." *Journal of Biomedicine and Biotechnology* 2011 (2011): 1-10.

Bershadsky, A. D., and Gelfand, V. I. "ATP-Dependent Regulation of Cytoplasmic Microtubule Disassembly." *Proceedings of the National Academy of Sciences* 78.6 (1981): 3610-613.

Cassimeris, L., Silva, V. C., Miller, E., Ton, Q., Molnar, C., and Fong, J. "Fueled by Microtubules: Does Tubulin Dimer/Polymer Partitioning Regulate Intracellular Metabolism?" *Cytoskeleton* 69 (2012): 133-43.

Chu, Chih-Wen, Fajian Hou, Junmei Zhang, Phu, L., Alex, V., Loktev, Donald, S., Kirkpatrick, Jackson, P. K., Yingming Zhao, and Hui Zou. "A Novel Acetylation of β-tubulin by San Modulates Microtubule Polymerization via Down-regulating Tubulin Incorporation." *Molecular Biology of the Cell* 22.4 (2011): 448-56.

Escuin, D., Kline, E. R., and Giannakakou, P. "Both Microtubule-stabilizing and Microtubule-destabilizing Drugs Inhibit Hypoxia-inducible Factor-1alpha Accumulation and Activity by Disrupting Microtubule Function." *Cancer Research* 65.19 (2005): 9021-028.

Friedman, J. R., B. M. Webster, D. N. Mastronarde, K. J. Verhey, and G. K. Voeltz. "ER Sliding Dynamics and ER-mitochondrial Contacts Occur on Acetylated Microtubules." *The Journal of Cell Biology* 190.3 (2010): 363-75.

Geeraert, C., Ratier, A., Pfisterer, S. G., Perdiz, D., Cantaloube, I., Rouault, A., Pattingre, S., Proikas-Cezanne, T., Codogno, P., and Pous, C. "Starvation-induced Hyperacetylation of Tubulin Is Required for the Stimulation of Autophagy by Nutrient Deprivation." *Journal of Biological Chemistry* 285.31 (2010): 24184-4194.

Gundersen, G. G., Khawaja, S., and Bulinski, J. C. "Postpolymerization Detyrosination of Alpha-tubulin: A Mechanism for Subcellular Differentiation of Microtubules." *The Journal of Cell Biology* 105.1 (1987): 251-64.

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, Mery, A., Vasquez, D. S., Turk, B. E., and Shaw, R. J. "AMPK Phosphorylation of Raptor Mediates a Metabolic

Checkpoint." *Molecular Cell* 30.2 (2008): 214-26.

Hawley, S. A. "Calmodulin-dependent Protein Kinase Kinase-b Is an Alternative Upstream Kinase for AMP-activated Protein Kinase." *Cell Metabolism* (2005): 9-19.

Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., and Hardie, G. D. "Characterization of the AMP-activated Protein Kinase Kinase from Rat Liver and Identification of Threonine 172 as the Major Site at Which It Phosphorylates AMP-activated Protein Kinase." *The Journal of Biological Chemistry* 271.44 (1996): 27879-7887.

Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Andrew, N., Yoshida, M., Xiao-Fan, W., and Tso-Pang, Y. "HDAC6 Is a Microtubule-associated Deacetylase." *Nature* 417 (2002): 455-58.

Infante, S. A., Stein, M. S., Zhai, Y., Borisy, G. G., and Gundersen, G. G., "Detyrosinated (Glu) Microtubules Are Stabilized by an ATP-sensitive Plus-end Cap." *Journal of Cell Science* 113 (2000): 3907-919.

Inoki, K., Zhu, T., and Kun-Liang, G. "TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival." *Cell* 115.5 (2003): 577-90.

Janke, C., and Bulinski, J. C. "Post-translational Regulation of the Microtubule Cytoskeleton: Mechanisms and Functions." *Nature Reviews Molecular Cell Biology* 12.12 (2011): 773-86.

Jaulin, F., and Kreitzer, G. "KIF17 Stabilizes Microtubules and Contributes to Epithelial Morphogenesis by Acting at MT plus Ends with EB1 and APC." *The Journal of Cell Biology* 190.3 (2010): 443-60.

Khawaja, S., Gundersen, G. G., and Bulinski, J. C. "Enhanced Stability of Microtubules Enriched in Detyrosinated Tubulin Is Not a Direct Function of Detyrosination Level." *The Journal of Cell Biology* 106.1 (1988): 141-49

Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J., and Winder, W.W. "5' AMP-activated Protein Kinase Activation Causes GLUT4 Translocation in Skeletal Muscle." *Diabetes* 48 (1999): 1-5.

LeDizet, M., and Piperno, G. "Cytoplasmic Microtubules Containing Acetylated A-Tubulin in Chlamydomonas Reinhardtii: Spatial Arrangement and Properties." *The Journal of Cell Biology* 103.1 (1986): 13-22.

Maruta, H., Greer, K., and Rosenbaum, L. J. "The Acetylation of Alpha-tubulin and Its Relationship to the Assembly and Disassembly of Microtubules." *The Journal of Cell Biology* 103.2 (1986): 571-79.

Matthias, P., Minoru, Y., and Khochbin, S. "HDAC6 a New Cellular Stress Surveillance Factor." *Cell Cycle* 7.1 (2008): 7-10.

Merrill, G. M., Kurth, E., Hardie, D. G. and Winder, W. W. AICAR decreases malonyl-CoA and increases fatty acid oxidation in skeletal muscle of the rat. *Am. J. Physiol.* (1997) 273, E1107-E1112.

Mihaylova, M. M., and Shaw, R. J. "The AMPK Signalling Pathway Coordinates Cell Growth, Autophagy and Metabolism." *Nature Cell Biology* 13.9 (2011): 1016-023.

Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D., and Kahn, B. B. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415, (2002). 339-343.

Nakano, A., Hisakazu, K., Takashi, W., Kyung-Duk, W., Satoru, Y., Yoshihiro, A., Seguchi, O., Higo, S., Shintani, Y., Asanuma, H., Asakura, M., Minamino, T., Kaibuchi, K., Mochizuki, Kitakaze, M., and Takashima, S. "AMPK Controls the Speed of Microtubule Polymerization and Directional Cell Migration through CLIP-170 Phosphorylation." *Nature Cell Biology* 12.6 (2010): 583-90.

Oakhill, J. S., Zhi-Pin, C., Scott, J. W., Steel, R., Castelli, L. A., Ling, N., Macaulay, L.S., and Kemp, B. E. "β-Subunit Myristoylation Is the Gatekeeper for Initiating Metabolic Stress Sensing by AMP-activated Protein Kinase (AMPK)." *PNAS* 107.45 (2010): 19237-9241.

Peris, L., Wagenbach, M., Lafanechere, L., Brocard, J., Moore, A. T., F. Kozielski, D., Job, L., Wordeman, and Andrieux, A. "Motor-dependent Microtubule Disassembly Driven by Tubulin Tyrosination." *The Journal of Cell Biology* 185.7 (2009): 1159-166.

Sanders, M. J., Ali, Z. S., Hegarty, B. D., Heath, R., Snowden, M. A., and Carling, D. "Defining the Mechanism of Activation of AMP-activated Protein Kinase by the Small Molecule A-769662, a Member of the Thienopyridone Family." *Journal of Biological Chemistry* 282.45 (2007): 32539-2548.

Scott, J. W., Bryce, J. W., Dendersen, V., Jorgensen, S. B., Honeyman, J. E., Steinberg, G. R., Oakhill, J. S., Iseli, T. J., Koay, A., Gooley, P. R., Stapleton, D., and Kemp, B. E.

"Thienopyridone Drugs Are Selective Activators of AMP-Activated Protein Kinase B1-Containing Complexes." *Chemistry & Biology* 15 (2008): 1220-230.

Verhey, K. J., and Gaertig, J. "The Tubulin Code." *Cell Cycle* 6.17 (2007): 2152-160.

Warden, S. M., Richardson, C., O'Donnell, J. Jr., Stapleton, D., Kemp, B. E., and Witters, L. A. "Post-translational Modifications of the B-1 Subunit of AMP-activated Protein Kinase Affect Enzyme Activity and Cellular Localization." *Biochemistry Journal* 354 (2001): 275-83.

Weinberg, R. A. *The Biology of Cancer*. New York: Garland Science, 2007.

Webster, D. R., Gundersen, G. G., Bulinski, J. C., and Borisy, G. "Differential Turnover of Tyrosinated and Detyrosinated Microtubules." *Proceedings of the National Academy of Sciences* 84.24 (1987): 9040-044.

Webster, D. R., and Borisy, G. G. "Microtubules Are Acetylated in Domains That Turn over Slowly." *Journal of Cell Science* 92 (1989): 57-65.

Weinberg, R. A. *The Biology of Cancer*. New York, NY [u.a.: GS Garland Science, Taylor & Francis Group, 2007

Witters, L. A., Kemp, B. E., and Means, A. R. "Chutes and Ladders: The Search for Protein Kinases That Act on AMPK." *Trends in Biochemical Sciences* 31.1 (2006): 13-16.

Wloga, D., and Gaertig, J. "Post-translational Modifications of Microtubules." *Journal of Cell Science* 124.1 (2010): 155

Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Lee, G. D., Neumann, F. D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D. "LKB1 Is the Upstream Kinase in the AMP-Activated Protein Kinase Cascade." *Current Biology* 13.22 (2003): 2004-008.

Xie, R., Nguyen, S., Mckeehan, W. L., and Liu, L. "Acetylated Microtubules Are Required for Fusion of Autophagosomes with Lysosomes." *BMC Cell Biology* 11.89 (2010): 1-12.