6-2016

# Tubulin Post-Translational Modifications are altered by changes in Actin-Myosin Contractility in non-muscle cells

James McGee
*Union College - Schenectady, NY*

Running Title: Tubulin Post-Translational Modifications are altered by changes in Actin-Myosin Contractility in non-muscle cells

# Tubulin Post-Translational Modifications are altered by changes in Actin-Myosin Contractility in non-muscle cells

By James P. McGee

* * * * * * * * * * *

Submitted in partial fulfillment of

the requirements for

Honors in the Department of Biological Sciences

UNION COLLEGE

June, 2016

**Abstract**

McGEE, JAMES Tubulin Post-Translational Modifications are altered by changes in Actin-Myosin Contractility in non-muscle cells
Department of Biological Sciences, June 2016

ADVISOR: Barbara A. Danowski, PhD.

All cells regulate their contractility by the interaction of actin and myosin. In non-muscle cells, however, this interaction is regulated by rho, a GTPase, which is the upstream effector for multiple pathways. Cell contractility increases when rho is active, and prior research has shown the depolymerization of microtubules (MTs) activates rho. Microtubules play a role in many cellular functions and it is thought that either associations with MT-associated proteins and/or post-translational modifications to tubulin regulate MT functions. Numerous post-translational modifications (PTMs) to microtubules have been identified, but their roles are not well understood. A possible relationship between changes in cell contractility and two tubulin post-translational modifications, acetylation and detyrosination, has been suggested by past research. We investigated if altered cell contractility caused changes in MT acetylation and detyrosination. We found that inhibition of rho caused a decrease in the amount of acetylation while activation of rho caused an increase. Since MT acetylation and detyrosination correlate with more stable MTs, our results suggest that a signaling pathway or feedback mechanisms exists in cells that allow them to respond to changes in contractility by altering MT dynamics. This not only sheds light on the crosstalk between actin and microtubules due to rho having a feedback mechanism as opposed to linear, but also allows for the speculation that one of the downstream effects of rho is the regulation of the enzymes that control MT acetylation.

**Introduction**:

The cytoskeleton of the cell is required for cells to alter their shape and size, to move and migrate, to interact within and outside of the cell, and to establish polarity. The cytoskeleton is also necessary for cell contractility, which is generated by the interaction of actin and myosin. Actin filaments are a main component of the cytoskeleton that are especially important in cell adhesion, movement, and migration. The myosin family, composed of actin-dependent motor proteins, interact with actin filaments and harness the power of ATP hydrolysis to cause cell movement. In non-muscle cells, the interaction between actin and myosin is regulated by phosphorylation of the myosin light chains, which is in turn influenced by a g-protein called rho (Alberts et al., 2009).

The Rho family of GTPases controls the organization and arrangement of the actin cytoskeleton, and includes many proteins including rac, rho, and cdc42. This Rho family of GTPases is able to be regularly studied due to the conservation of these proteins in organisms throughout evolution (Rajas et al., 2015). GTPases act as molecular switches by becoming active when bound to GTP and inactive when the GTP is hydrolyzed to GDP. One of these family members, rho, controls and modulates cell contractility and adhesion plaque formation through its many downstream effectors. One of the rho pathways promotes cell contractility (reviewed by Riching and Keely, 2015). In this pathway, when rho becomes active once bound to GTP, a downstream effector ROCK, or rho kinase, becomes activated. When active, ROCK has been found to both phosphorylate and activate myosin light chains and to phosphorylate, and therefore inhibit the myosin light chain phosphatase, and therefore keep the myosin light chains active (Figure 1). When phosphorylated, the myosin light chains allow the entire myosin motor protein to complete cycle movements along the actin filament, allowing for cellular contractility. When bound together, this association of actin filaments, myosin, and other proteins form actin stress fibers that stretch across the entirety of the cell to adhesion

plaques, which are sites in the membrane of a cell that contain proteins that function to keep the cell adhesive to the extracellular matrix or other surfaces. Furthermore, it is known that when rho is inhibited actin stress fibers and adhesion plaques disassemble (Alberts et al., 2009).

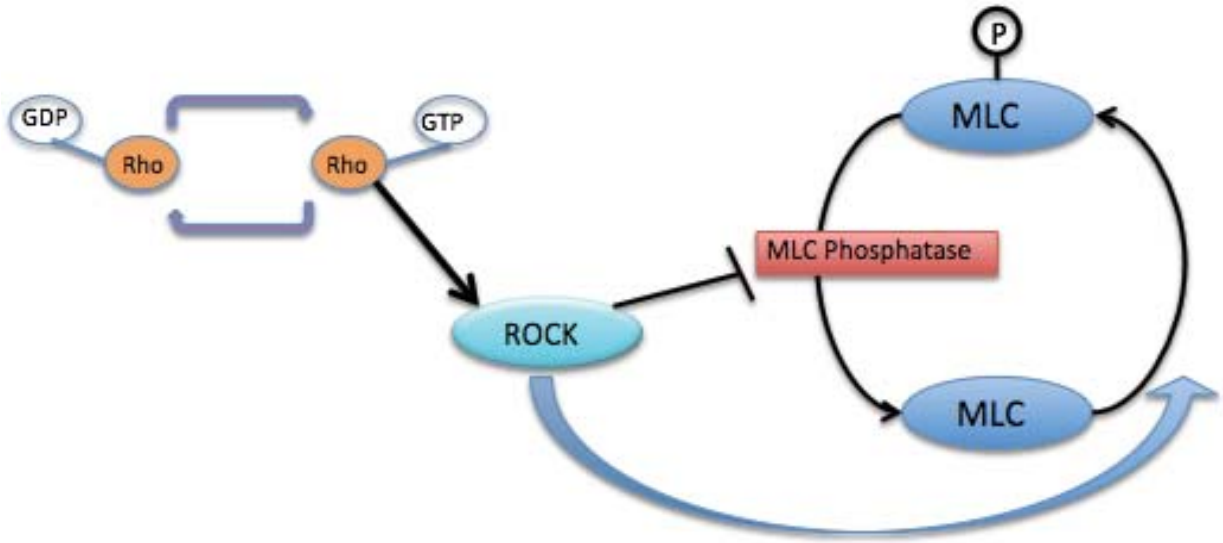



**Figure 1:** Diagram of the actin-myosin contractility rho pathway

Another component of the cell's cytoskeleton, microtubules (MTs), contribute to this pathway. Microtubules play roles in various cell functions including chromosome segregation, establishment of polarity, vesicle transport, and creating permanent structures to create cilia and flagella. MTs are hollow tubes formed by polymers composed of the two subunits, alpha-tubulin and beta-tubulin, and have a unique characteristic known as dynamic instability. Dynamic instability allows for MTs to abruptly switch from polymerizing (lengthening) to depolymerizing (shortening) or neither, a state where the MT is considered stable (Figure 2). These changes can occur to multiple or individual MTs at any given time and contribute to cell contractility in doing so (Alberts et al., 2009). When more microtubules are stabilized, the microtubules throughout the entire cell are considered less dynamic. Microtubules have also been

shown to play a role in the regulation of actin functions by crosstalk between the two components of the cytoskeleton. (Alberts et al., 2009).

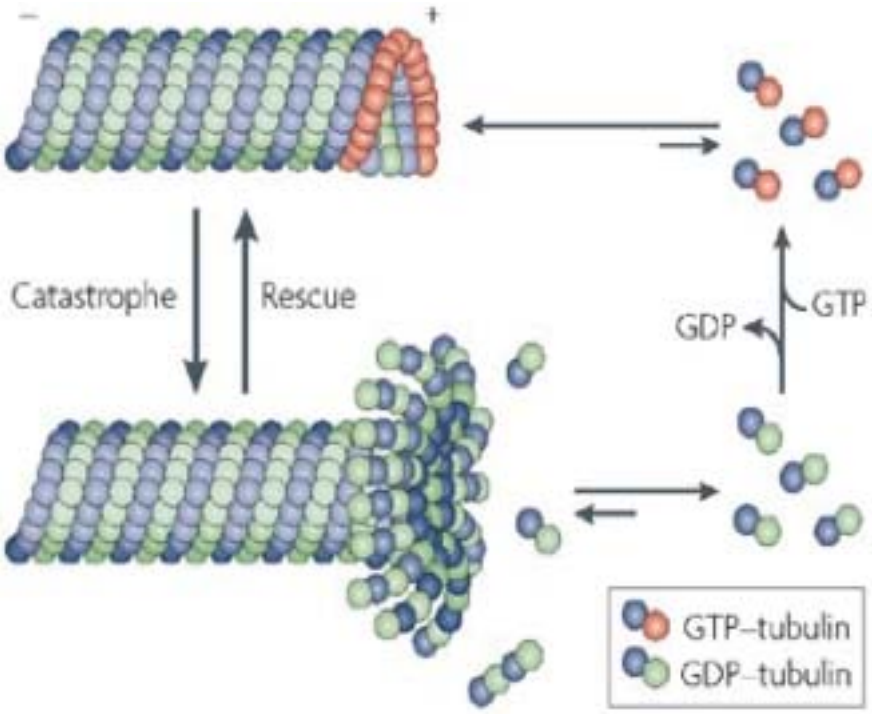



**Figure 2**: Diagram of microtubule dynamic instrability (Reviewed by Cheeseman, IM, and Desai, A. "Molecular architecture of thje kinetochore-microtubule interface" Nat Rev Mol Cell Biol. 9 (1):33-46. 2008)

The concept of dynamic instability with MTs is not entirely understood and it is widely thought that post-translational modifications (PTMs) to MTs play a significant role. Two of the more studied microtubule PTMs are acetylation and detyrosination, and detyrosination has been found to be correlated with MT stability (reviewed by Westermann and Weber, 2003). Detyrosination is the removal of the C-terminal amino acid tyrosine from an alpha-tubulin dimer via a carboxypeptidase enzyme and it only occurs on polymerized MTs, for upon depolymerization the dimers are re-tyrosinated (Gundersen et al., 1987). Detyrosination has been found to be a marker in determining the length of time a MT has remained polymerized, or stable, rather than occurring to stabilize a MT (reviewed by Westermann and Weber, 2003). Acetylation is the addition

of an acetyl group to a lysine amino acid on the alpha-tubulin on the inside face of the microtubule (Figure 3). Histone deacetylase 6 (HDAC6) and SIRT2 have been identified as the two deacetylating enzymes, while alpha-TAT1 is the acetylase (reviewed by Janke and Bulinski, 2011).

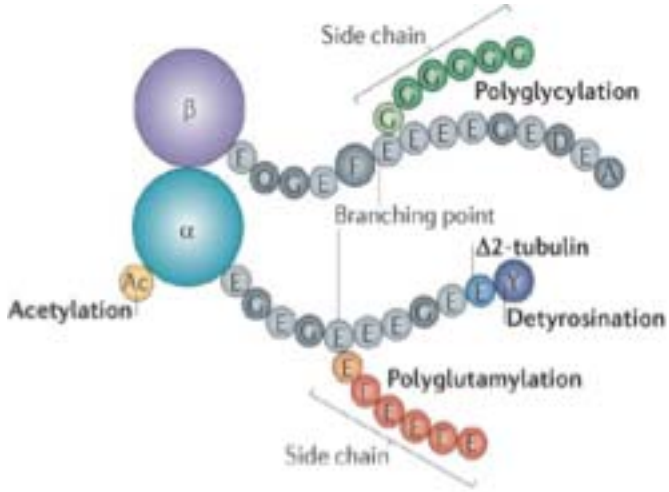



**Figure 3:** Diagram of microtubule post-translational modifications (Reviewed by Janke and Bulinski, 2012)

Previously in the Danowski Lab, microtubules in C3H10T½ mouse embryo fibroblasts subjected to stress by ATP depletion have been observed to become stable, as determined by the inability of nocodazole to depolymerize the MTs. MTstability was correlated with an increased amount of detyrosinated tubulin and acetylated tubulin. Recently, other stresses, including amino acid starvation and UVC radiation, were shown to increase the amount of MT acetylation and detyrsoination, compared to control cells (Mackeh et al., 2014). Furthermore, it has also been observed that increased MT deacetylation due to the activation of histone deacetylase 6 (HDAC6), microtubules are found to promote more rapid depolymerization. This suggests that acetylation to microtubules decreases microtubule dynamics and plays a role in keeping them polymerized (Nam et al., 2010).

It is known that microtubules influence the contractility of the actin cytoskeleton. For example, when microtubule depolymerization was induced by drugs such as

nocodazole, vinblastine, or colcemid, non-muscle cells responded by forming stress fibers and focal adhesions (Danowski, 1989). This observation led to the idea of crosstalk between the two components of the cellular cytoskeleton in a mechanistic way to balance the compressive and tensile forces within the cell (Danowski, 1989). Danowski (1989) also suggested the possibility that MTs sequester a contractility-stimulation factor, that is released upon MT depolymerization. It was subsequently shown that rho activation initiated the pathway to induce cell contractility, while microtubule depolymerization has also been seen to induce increased cell contractility, so the relationship was investigated and it was observed that the depolymerization of microtubules does in fact activate rho (Liu et al., 1997). Chang and colleagues (2008) showed that upon depolymerization, MTs release GEF-H1, a microtubule associated guanine nucleotide exchange factor known to activate rho. If the depolymerization of MTs activates rho, and increases cell contractility, it is possible that the inhibition of cell contractility would cause a feedback mechanism to alter the microtubules in some way., possibly through MT post-translational modifications. We sought to investigate whether evidence of such a feedback mechanism exists by examining the effects to microtubules upon altering the rho pathway.

Another example of intersection between microtubules and cell contractility involves the regulation of HDAC6 by tubulin polymerizing protein 1 (TPPP1). Schofield et al. (2012) found that TPPP1 binds to HDAC6, and this binding prevents HDAC6 activity. However, when TPPP1 is phosphorylated, HDAC6 is released and causes a decrease in MT acetylation. The kinase responsible for phosphorylating TPPP1 is ROCK, one of the downstream effectors of rho. Thus, they concluded that activation of the rho signaling pathway can lead to alterations in MT acetylation, mediated through TPPP1 (Schofield et al. 2015). mDia2 has been discovered to cause changes to microtubule detyrosination. mDia1 and mDia2 are two diaphanous-related formins that are involved in the capping

(stabilizing) and the capture (depolymerizing) of microtubules. mDia2 was found to be another downstream effector of rho, and when activated separately mDia2 caused an increase in the amount of detyrosinated, and therefore assumed stable, microtubules (Palazzo et al., 2001). We therefore hypothesized that if rho was inhibited, a significant decrease to the amount of detyrosinated microtubules would be observed.

Due to this research we hypothesized that there was some form of feedback mechanism in this cell contractility to microtubules that could be observed through looking at post-translational modifications to microtubules. To investigate this, C3H10T½ mouse embryonic fibroblast cells will be used to observe the relationships of actin and microtubules as well as microtubules and cell contractility. This rho pathway will be studied through using a membrane permeant rho inhibitor, ROCK inhibitor, and rho activator and examined using immunofluorescence microscopy and western blotting. In using antibodies to stain for alpha tubulin, acetylated tubulin, and detyrosinated tubulin, the localization, amount, and concentration of both microtubules and the post translational modifications can be analyzed. The results could shed further light on the mechanisms of microtubule-actin cytoskeletal crosstalk, provide more insight into the role of acetylated and detyrosinated microtubules, and explore potential unknown downstream pathways of rho.

**Methods**:

*Immunofluorescence Microscopy*:

C3H10T½ mouse embryo fibroblasts were grown in a twelve well dish on small cover slips to minimize the volume of medium used. Besides the control cells, cells were treated with the following membrane permeant treatments: 10uM rho inhibitor, 10 uM ROCK inhibitor, and 20 uM rho activator (all from Cytoskeleton, Inc.). The rho inhibitor was used in a time course for one, four, and twelve hours, and then in multiple experiments for one and four hours. Cells were treated with ROCK inhibitor for one hour and the rho activator for two hours. Cells were then fixed in 99% pure ice cold methanol for ten minutes. Coverslips were then air dried for five minutes, followed by five washes in calcium and magnesium free phosphate buffered saline (CMF-PBS). Coverslips were then incubated with a primary antibody for either two hours at 37°C in a moist chamber or three hours at room temperature (mouse anti-acetylated tubulin, 1:200; anti-rabbit detyrosinated tubulin, 1:150; mouse DM1A for alpha-tubulin, 1:100, aka DM1A). After repeating fives washes in PBS, coverslips were incubated in secondary antibody for either two hours at 37°C in a moist chamber three hours at room temperature (goat anti-mouse Alexa Red 1:300; goat anti-mouse Alexa Green 1:300). For acetylated tubulin, another wash of five times in PBS was done. Then mouse FITC-DM1A anti-total alpha-tubulin at 1:100 was added to the coverslips and was incubated in a moist chamber for either two hours at 37°C er or three hours at room temperature. After all incubations were complete, the coverslips were washed four times in PBS and a fifth time in Milli-Q water. The coverslips were then mounted on slides with ProLong Gold Anti Fade w/ DAPI stain. Note: all of the antibody dilutions were done with PBS.

*Preparation of Total Cell Lysate*:

C3H10T½ mouse embryo fibroblasts were grown in 60mm sized plates until fully dense. Besides a control plate with no drugs added, the plates were treated with either a 10uM rho inhibitor for one hour, a 10 uM ROCK inhibitor for one hour, or 20 uM rho activator for two hours. Cell lysates were made from these dishes. To do so, the dishes were rinsed with warm PBS twice prior to the addition of ice cold RIPA buffer (200 ul) and protease inhibitor cocktail (PIC) (1 ul PIC/100ml RIPA buffer). The cells were scraped off the plate while keeping it on ice and the liquid containing the cells was transferred to a pre-chilled 1.5 ml microfuge tube. While at 4°C, the tubes were mixed for 15 minutes and then spun at 12,000 for 5 minutes. After 10ul of each tube was taken to measure the protein concentration via nanodrop, the remaining supernatant was transferred to another pre-chilled tube and an equal amount of 2x sample buffer with DTTP was added. The pellet from the original tube was then disregarded. The lysates were then boiled for 6 minutes prior to storage at -20°C. The lysates were thawed when needed for use.

*Western Blot*:

Pre-cast ten-well Mini-Protean BioRad® 4-15% gels were used for acetylated tubulin and total tubulin, lower molecular weight proteins. BioRad® Dual Precision Plus was loaded into a well next to the cell lysates. Gel electrophoresis was run at room temperature for approximately 45 minutes (90V until the samples left the wells and then upped to 150V) in running buffer (3 g TRIS base, 14.4 g glycine, pH 7). At the end of electrophoresis a PVDF membrane was rinsed in room temperature 99% methanol and washed in transfer buffer for ten minutes. The gel from electrophoresis was also washed in transfer buffer for ten minutes (100ml 10X Running Buffer, 1ml 10% SDS/1 liter; 20% methanol/1 liter). Once the washes were done, the proteins from the gel were

transferred onto the PVDF membrane using a semi-dry blotter. Two thick pieces of blotting paper were cut to sandwich in the gel and membrane and also soaked in transfer buffer (100ml 10X Running Buffer, 1ml 10% SDS/1 liter; 20% methanol/1 liter). The gel was placed closer to the cathode and the membrane to the anode, and placed in the semi-dry blotter. This was run at 15V for 20 minutes. After the transfer was complete the PVDF membrane was washed in blocking buffer for four hours (1 part powdered milk/20 parts TTBS). The membrane was then washed 5 times at 4 minutes each in TTBS (TrisBuffered Saline (BioRad) + 0.05% Triton X-100) while rocking. The membrane was then incubated with the primary antibodies overnight at 4°C (anti-acetylated tubulin made in mouse 1:2,500; anti-alpha tubulin made in mouse 1:5,000; and anti-GAPDH made in mouse 1:3,000) in 10ml TTBS (TrisBuffered Saline (BioRad) + 0.05% Triton). The next morning the membrane was washed 5 times at 4 minutes each in TTBS. After the washes, the PVDF membrane was incubated in secondary antibodies for two hours at room temperature (1:10,000 goat anti-mouse HRP) and then subjected to one last round of 5 washes at 4 minutes each. Then the PVDF membrane was developed with Femto Developing Solution by Piercenet ®. The western blots were quantified by the BioRad ® Image Lab.

**Results:**

Rho Inhibitor disrupts actin stress fibers in mouse embryonic fibrobasts

In C3H10T½ mouse fibroblast cells, the membrane permeant rho inhibitor was successfully able to disrupt the actin stress fibers in the treated cells compared to the control cells. After one hour of treatment with the rho inhibitor, a significant decrease in the amount of stress fibers and thickness of the stress fibers was observed in the majority of the cells through immunofluorescence microscopy. After four hours the most significant disruption of actin stress fibers was seen, along with the delocalization of paxillin, a adhesion plaque protein found at the termini of stress fibers (Figure 4). Although these changes did not occur to every cell, they were observed in the majority of the cells treated with the rho inhibitor compared to the control cells.

Does the inhibition of rho cause any change to the localization or amount of acetylated microtubules?

C3H10T½ fibroblast cells were subjected to either one hour, four hours, or no treatment with the membrane permeant rho inhibitor. The amount of acetylated microtubules in control cells varied considerably. In order to detect changes induced rho inhibition, cells stained with anti-acetylated tubulin antibody were evaluated visually and categorized as having less/decreased, moderate, or more/increased amounts of acetylation to microtubules (Figure 5). This categorization was applied to those cells treated with the rho inhibitor for one hour and four hours and after three experiments the data was quantified. After one and four hours of rho inhibition, there was a decrease in the acetylated MTs, as seen by the relative increase in the number of cells categorized as having lesser amounts of acetylated MTs. The differences in the number of cells in the lesser amounts of acetylated MTs between the control cells and those treated with rho inhibitor for one hour, and the control cells and those treated with the rho inhibitor for

four hours were significant (P=0.002 and P=0.003, respectively). Therefore, it was determined that the inhibition of rho significantly decreases the amount of acetylation to microtubules.

Does the inhibition of rho cause any change to the localization or amount of detyrosinated microtubules?

A similar analysis was performed to investigate any changes in microtubule detyrosination in response to rho inhibition. Although there was a slight increase in the number of cells showing moderate amounts of MT detyrosination, the differences were not significant (Figure 6). Therefore, it was determined that the inhibition of rho did not have any effect on the localization or amount of detyrosination.

Can changes in acetylated tubulin in response to altering rho activity be detected by western blotting?

The western blot done shows the lysates of control C3H10T½ cells, those treated with the rho inhibitor for one hour, ROCK inhibitor for one hour,  and rho activator for two hours (Figure 7). Lysates of cells treated with the rho inhibitor for one hour contained less acetylated tubulin compared to the control cells. The control protein band, GAPDH, was not of equal concentration, so the BioRad Imaging Program was used to ratio the amount of GAPDH to acetylated tubulin. It was found that rho inhibition reduced the amount of acetylated tubulin by 40%, but surprisingly ROCK inhibition had no effect. Activation of rho increased tubulin acetylation by 100% (Table 1).  analyze and compare the concentrations of the GAPDH to Acetylated Tubulin. The ratios given showed that while the cells treated with the ROCK inhibitor had no difference compared to the control, the cells treated with the rho inhibitor had less acetylated tubulin than the control and the cells treated with the rho activator had more acetylated tubulin (Table 1).

Does rho activation increase the amount of acetylated microtubules in cells?

C3H10T½ fibroblast cells were then stained for immunofluorescence microscopy after undergoing one hour treatment with the rho inhibitor, one hour treatment with the ROCK inhibitor, two hour treatment with the rho activator, or did not undergo treatment. The staining of these cells was observed and then categorized into those with moderate acetylation, those with less/few acetylation, and those with increased/higher acetylation. After three experiments the data was quantified. These results show the same results as those seen with the western blot, with rho inhibited cells having less acetylation than the control, ROCK inhibited cells not differing from the control cells in acetylation, and rho activated cells having more acetylation than the control (Figure 8). The difference in percentage of cells with less/fewer acetylation between the control cells and those treated with the rho inhibitor was found significant ($P=0.001$) and as well as those cells with moderate amounts of acetylation ($P=0.02$). The decrease in the amount of acetylated microtubules when rho is inhibited was again seen significant. Furthermore, the difference in percentage of cells with increased/more acetylation between the control cells and those treated with the rho activator was found to be significant ($P=0.008$) and as well as those cells with less/fewer amounts of acetylation ($P=0.0007$). The increase in the amount of acetylated microtubules when rho is activated was therefore found significant.

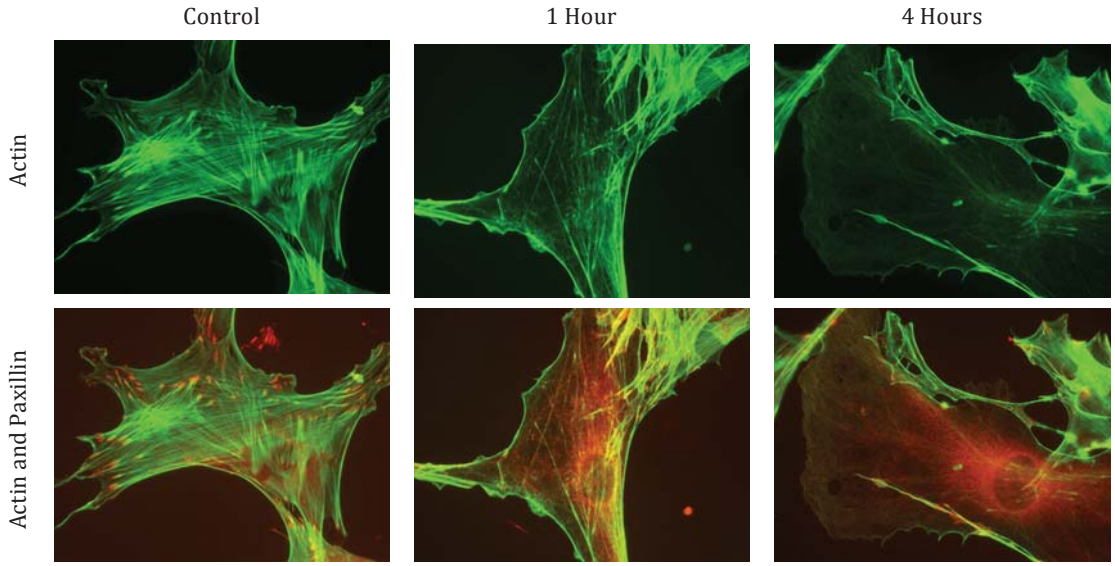
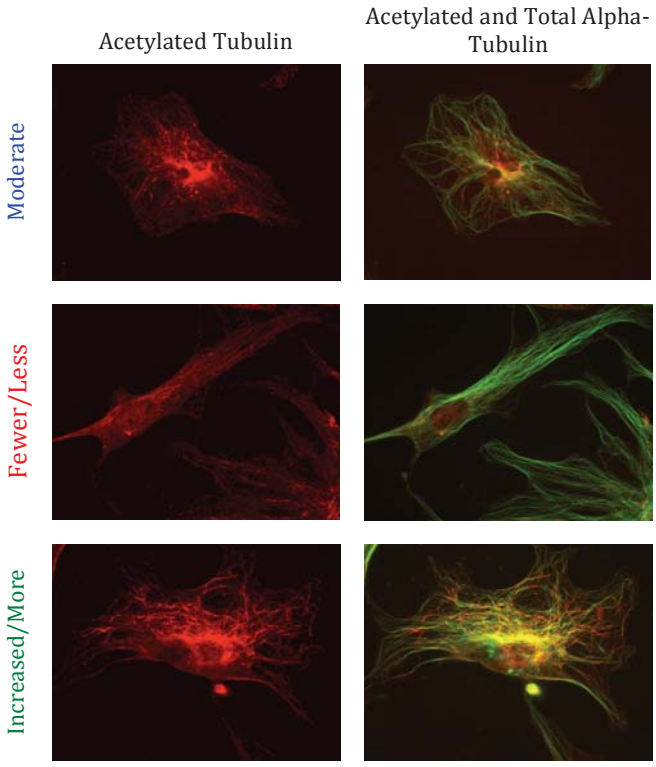



**Figure 4**: Immunofluorescence microscopy of the localization of actin stress fibers (green) and the paxillin, a protein within focal adhesions, (red) of cells were be observed. For both the one hour and four hour treatments, stress fibers were disrupted and paxillin was delocalized from adhesion plaques compared to the control.

A)




B)

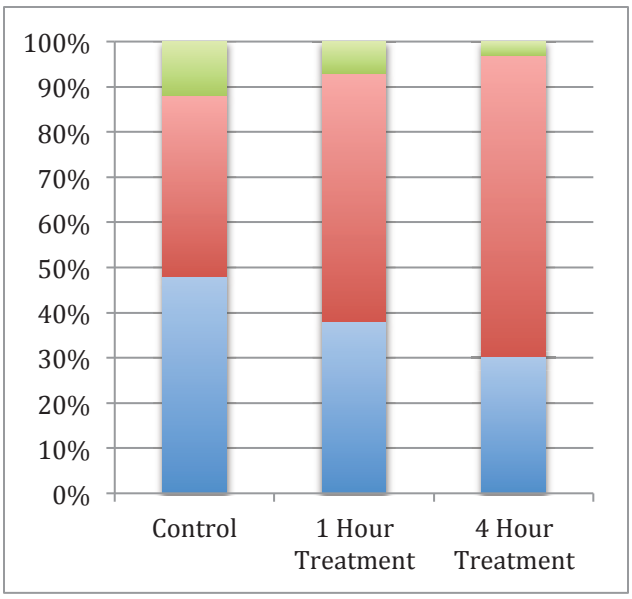
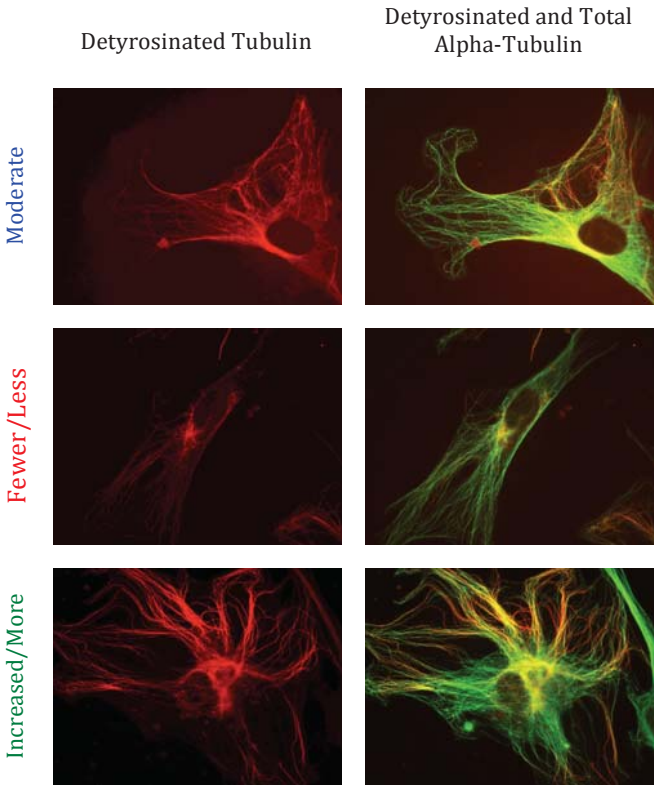



**Figure 5:** Cells stained with an antibody to alpha tubulin (green) and acetylated tubulin (red) treated with rho inhibitor for one hour, four hours, and those without treatment. A) Analysis of the percentage of cells showing fewer, moderate, and increased amounts of acetylation on their MTs.B) Percentages of the categories of cells with moderate (blue), fewer/less (red), and increased/more (green) for the control, one hour treatment, and four hour treatment. Means were gained over three experiments.

A)




B)

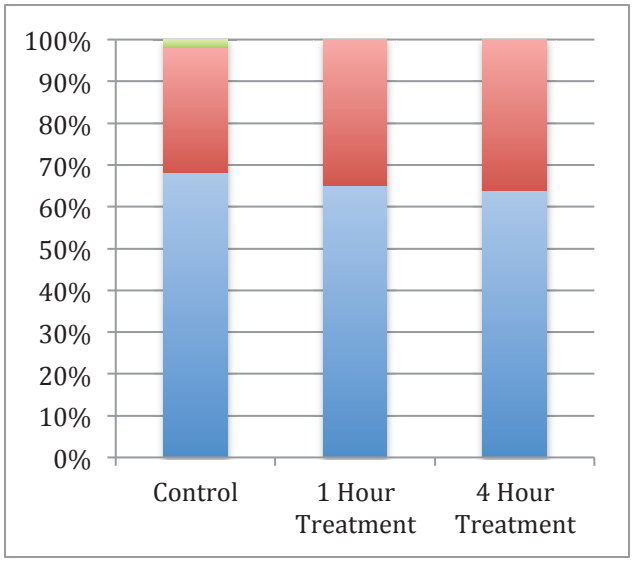



**Figure 6**: Cells stained with an antibody for alpha tubulin (green) and detyrosinated tubulin (red) treated with rho inhibitor for one hour, four hours, and those without treatment. A) Analysis of the percentage of cells showing fewer, moderate, and increased amount of acetylation on their MTs. B) Percentages of the categories of cells with moderate (blue), fewer/less (red), and increased/more (green) for the control, one hour treatment, and four hour treatment. Statistics were gained over three experiments.

**Table 1**: Quantification of Protein Concentrations from Western Blot

| Protein | Control | Rho Inhibitor | ROCK Inhibitor | Rho Activator |
|---|---|---|---|---|
| Ratio: Acetylated Tubulin/GAPDH | 0.18 | 0.11 (40% Decrease) | 0.18 | 0.35 (100% Increase) |

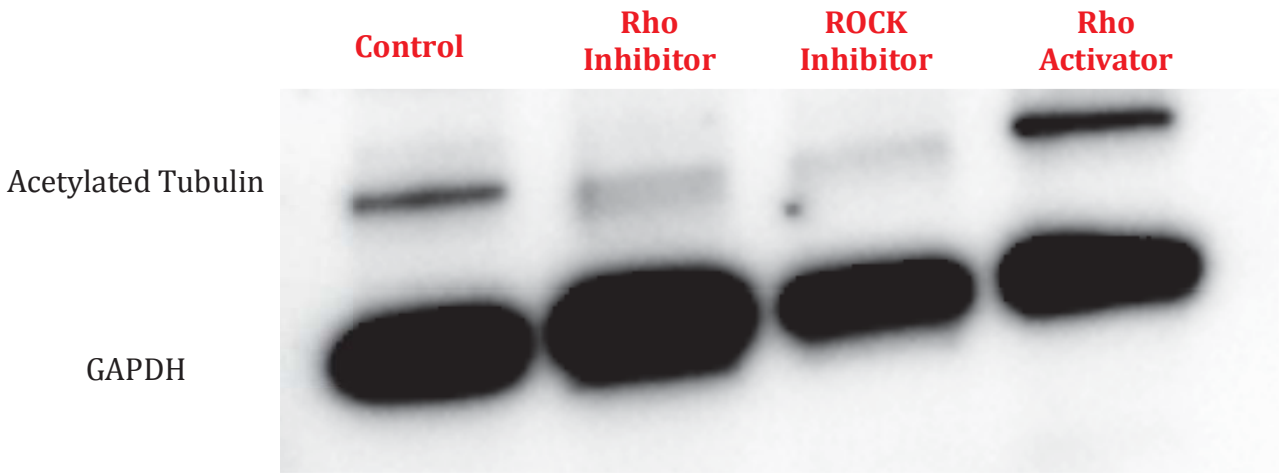



**Figure 7**: Imaged blot of lysates from cells treated with rho inhibitor for one hour, ROCK inhibitor for one hour, rho activator for 2 hours, or control cells. The ratio of acetylation to GAPDH was determined (Table 1).

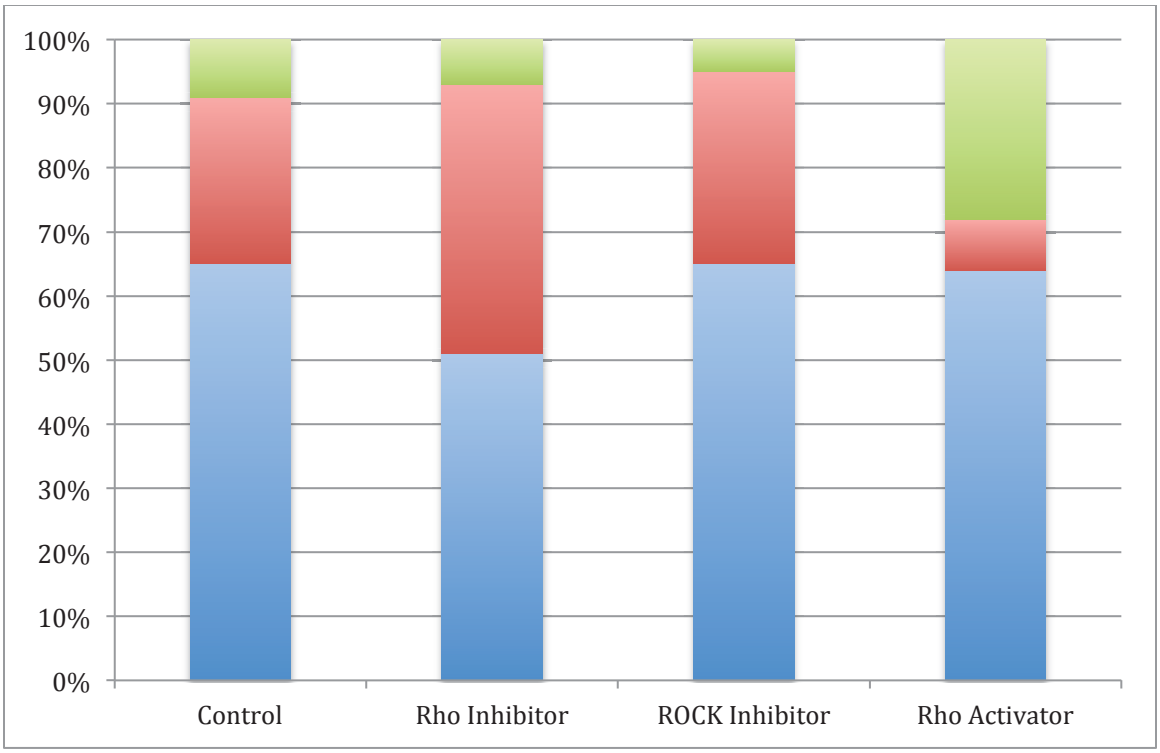



**Figure 8:** Graphical representation of cells that were either treated with a rho inhibitor for one hour, ROCK inhibitor for one hour, rho activator for two hours, or not treated. Immunofluorescence microscopy was used and cells were stained for total tubulin and acetylated tubulin. Cells were categorized into having moderate (Blue), fewer/less (Red), or more/increased (Green) amounts of acetylated tubulin.

**Discussion:**

We expected to see an increase in tubulin acetylation upon rho inhibition and found the opposite. Our expectation was based on the hypothesis that there is a reciprocal relationship between cell contractility and microtubule stability. Since MT depolymerization increases cell contractility (Danowski, 1989) via rho activation (Liu et al., 1998); we hypothesized that if a reciprocal relationship existed, a decrease in contractility would lead to more stable MTs. And stable MTS have been found to have more acetylation (Li et al., 2015) and detyrosination (reviewed by Westermann and Weber, 2003). One possible explanation for the increase in MT acetylation is that the rho inhibitor did not work properly, but analysis of f-actin in rho inhibited cells showed the characteristic loss of stress fibers as expected (Alberts et al., 2009).

There were no significant changes to the amount of detyrosination to microtubules when rho was inhibited. Detyrosination has been found to tag or occur to stable microtubules, and so we concluded that the inhibition of rho does not cause the stabilization of microtubules. This contrasts recent research where the inhibition of rho was found to increase the amount of detyrosination, and therefore microtubule stability, in NH-3T3 cells. The detyrosination was shown to be regulated by mDia2, and the upstream effector of mDia2 is rho (Palazzo et al., 2001). This research showed that rho is the upstream effector for other cell regulatory pathways as well for the activation of myosin-light chains. Our methodology for determining changes in MT detyrosination only looked at the localization of the detyrosination and not the total amount of detyrosinated MTs. Western blot analysis for detyrsoinated tubulin should have been done to confirm that in C3H10T½ cells no change to detyrosinated MTs occurred.

There was a change observed to the amount of microtubule acetylation when rho was inhibited, suggesting that a feedback mechanism exists. Acetylation has been seen to occur on microtubules in cells that have been put under stress and when treated with

drugs to depolymerize the microtubules, nocodazole, the microtubules remain stable. There was a significant decrease observed in the amount of acetylated microtubules in cells with inhibited rho observed in both immunofluorescent microscopy and western blotting. We speculate that the decrease in acetylated microtubules is representative of an increase in microtubule dynamics and is reflective of the fact rho is an integrator of many signals and pathways, therefore making the output hard to predict.

It has become increasingly clear that rho activation leads to a variety of downstream effectors, sometimes even seemingly opposite effects. For example research by Naranatt et al. (2004) and Destaing et al. (2004) shows contrasting results with each other and our results. The changes in acetylation to MTs when the contractility pathway has been altered have been studied in different cell lines with differing results. When the human Herpesvirus-8 (HHV-8) was introduced into human fibroblast cells, dermal microvascular endothelial cells, and embryonic kidney cells, hyperacetylation was observed. One of the many functions of this virus is its integrin interaction with ligands that control the cytoskeleton remodeling system, showing that a suggested manner in which virus works is rho dependent. Naranatt et al. (2004) found that rho inhibition of HHV-8 infected cells decreased acetylation tubulin. While Destaing et al. (2004) found an increase in tubulin acetylation in rho-inhibited osteoclasts. The cells were interestingly found with no differences in detyrosinated MTs, similar to our results but in contrast to Palazzo et al. (2001). Using exoenzyme C3 fused to the HIV TAT protein fragment to inhibit rho activity and then treating the osteoclasts with nocodazole, it was observed that cells treated with the rho inhibitor were able to resist nocodazole depolymerization for a significantly longer amount of time than the control cells. With the MTs proven to be stabilized, the amount of acetylation and detyrosination to tubulin was studied. Osteoclasts treated with the rho inhibitor had significantly more acetylated tubulin than the control cells, yet no difference in the amount of detyrosination was

observed (Destaing et al., 2004). Therefore, it can be determined that this crosstalk of the response in regards to the action of acetylation enzymes must be finely tuned to specific cell types, most likely due to their function, and that there is no direct connection between rho and the acetylation of MTs. Rho is instead an integrator of many signals and pathways, and it is unknown what downstream pathway gets activated to acetylate MTs or effect other post-translational modifications.

Knowing that microtubule depolymerization activated rho (Liu et al., 1997), we investigated what would occur if rho was activated via a membrane permeant rho activator as opposed to activating rho by depolymerizing the microtubules. Rho activation led to an increase of microtubule acetylation that was observed through immunofluorescence microscopy and western blotting, showing the exact opposite of when rho is inhibited. This allowed us to speculate that this relationship between cell contractility and microtubule acetylation was reciprocal. This increase in acetylation to microtubules was due to either the decrease in the activity of HDAC6 or an increase in the activity of the enzymes that acetylate microtubules such as alpha-tubulin acetyltransferase. In increasing acetylation, we can speculate that the dynamics of the microtubules are decreasing, which would work to prevent microtubules from depolymerizing, which could be a method of preventing further activation of rho (Figure 9).

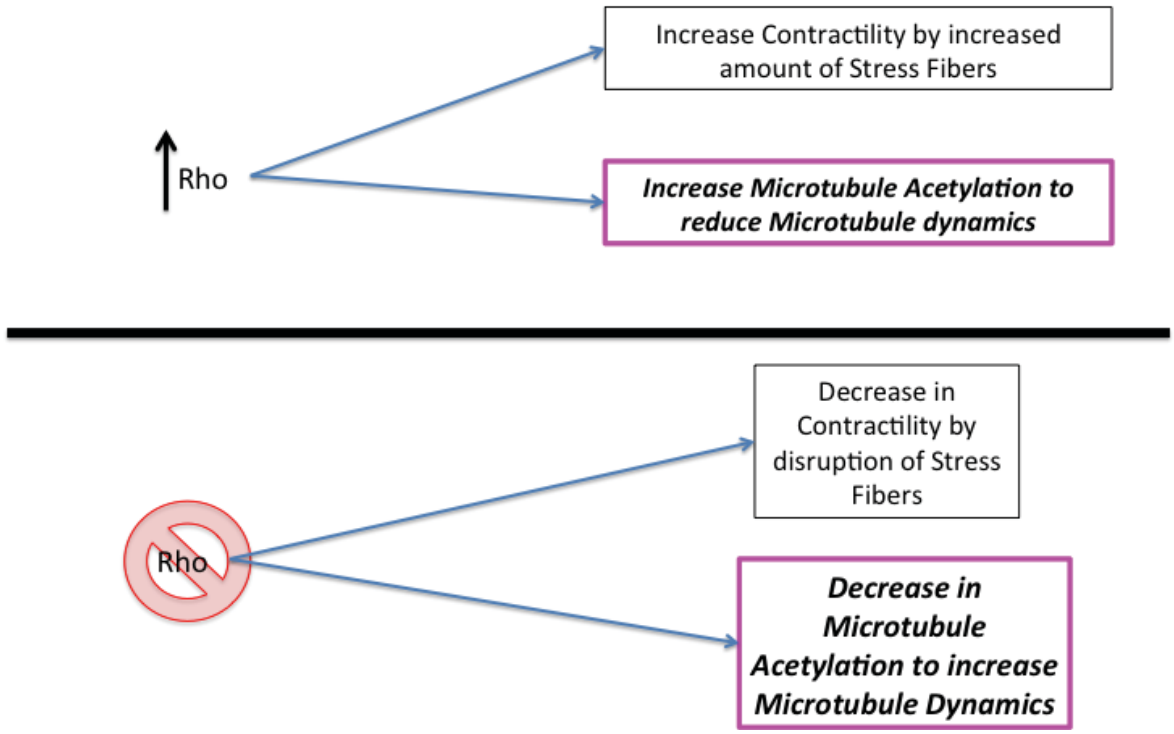



**Figure 9**: Working model for the relationship between microtubules and cell contractility

The downstream effector of this pathway, ROCK, was also investigated. So far the only changes in rho activity had been observed with the assumption that the changes to the amount of microtubule acetylation was because of the decrease or increases in cell contractility. Yet, what if this was instead due to an unknown downstream effector of rho (Figure 10)? Therefore, the inhibition of rho kinase, ROCK, was observed using both immunofluorescence microscopy and western blotting. Surprisingly there was no change in the amount of acetylation to microtubules observed through both immunofluorescence microscopy and western blotting, which led to the conclusion that rho is the only member of the rho pathway that is somehow able to regulate the acetylation to microtubules. Much like how rho was discovered to be the upstream effector to mDia2 activation to lead to an increase in detyrosination, rho must be the upstream effector in an undiscovered pathway that activates a regulator of acetylation to microtubules (Palazzo, A.F. et al., 2001). This pathway would allow for the

increased activity of HDAC6 to decrease acetylation when rho is inhibited or the activation of enzymes that acetylate microtubules when rho is activated.

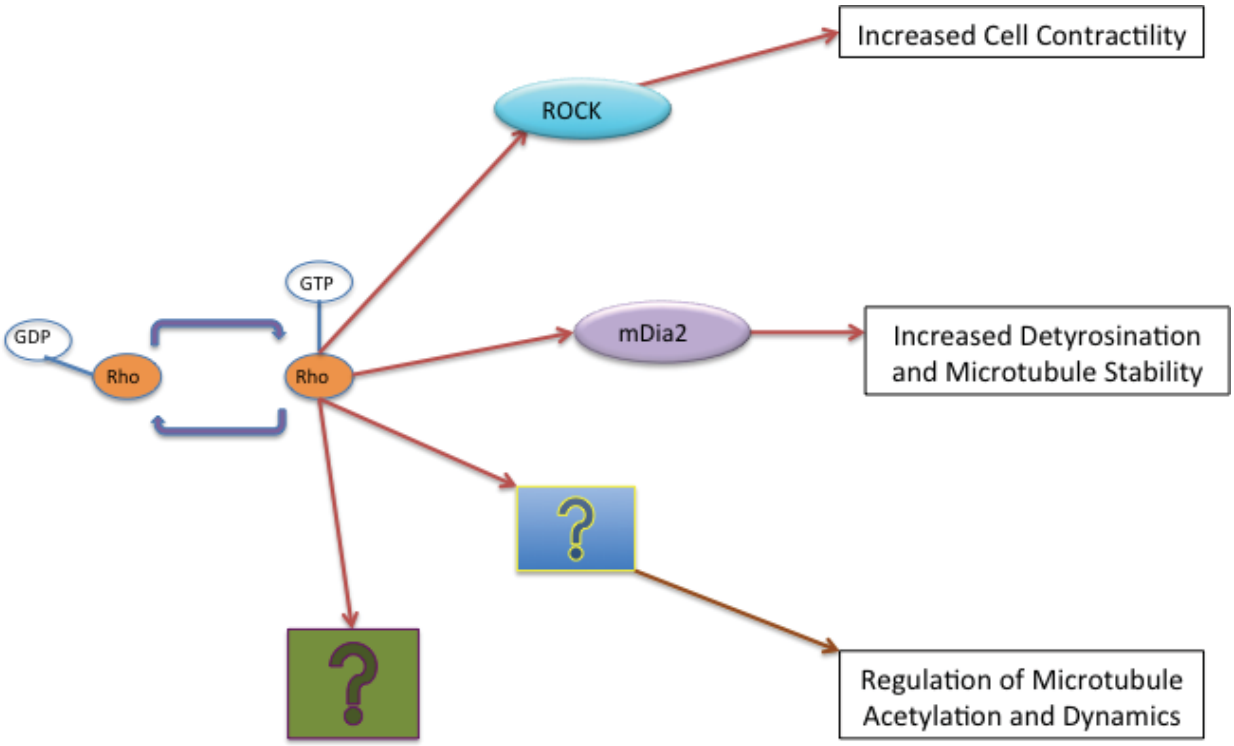



**Figure 10**: Working model for possible downstream effectors of rho

Works Cited

Alberts, Bray, Hopkin, Johnson, Lewis, Raff, Roberts, Walter. (2009) *Essential Cell Biology*. New York: Garland Science

Chang, Y., Nalbant, P., Birkenfeld, J., Chang, Z., Bokoch, G. (2008) “GEF-H1 Couples Nocodazole-induced Microtubule Disassembly to Cell Contractility via RhoA” *Molecular Biology of the Cell*. 19: 2147-2153

Danowski, Barbara. (1989) “Fibroblast contractility and actin organization are stimulated by microtubule inhibitors”. *J. Cell. Sci*. 93: 255-266

Destaing, O., Saltel, F., Gilquin, B., Chabadel, A., Khochbin, S., Ory, S., Jurdic, R. (2005) “A novel Rho-mDia2-HDAC6 pathway controls podosome patterning through microtubule acetylation in osteoclasts”. *Journal of Cell Science*. 118: 2901-2911

Gunderson, Gregg G., Sadiqa Khawaja, and Jeannette C. Bulinksi. (1987) "Postpolymerization Detyrosination of α-Tubulin: A Mechanism for Subcellular Differentiation of Microtubules." *The Journal of Cell Biology* 105: 251-64

Janke, Carsten, and Jeannette C. Bulinksy. (2011) "Post-translational Regulation of the Microtubule Cytoskeleton: Mechanisms and Functions." *Nature Reviews: Molecular Cell Biology* 12: 773-86

Li, L., Yang, X.(2015) “Tubulin acetylation: responsible enzymes, biological functions and human diseases.” Cell. Mol. Life Sci., 72: 4237-4255

Liu, B. P., Chrzanowska-Wodnicka, M., Burridge, K. (1998) “Microtubule depolymerization induces stress fibers, focal adhesions, and DNA synthesis via the GTP-binding protein Rho”. *Cell Adhes Commun*. 5(4): 249-255

Mackeh, R., Lorin, S., Ratier, A., Mejdoubi-Charef, N., Baillet, A., Bruneel, A., Hamai, A., Codogno, P., Pous, C., Perdiz, D. (2014) “Reactive Oxygen Species, AMP-activated Protein Kinase, and the Transcription Cofactor p300 Regulate alpha-

Tubulin Acetyltransferase-1 (alpha TAT-1/MEC-17)- dependent Microtubule Hyperacetylation during Cell Stress". *Journal of Biological Chemistry* 287(17): 11816-11828

Nam, H.J., Kang, J.K., Kim, S., Ahn, K.J., Seok, H., Park, S.J., Chang, J.S., Pothoulakis, C., Lamont, J.T., Kim, H. (2010) "Clostridium difficile Toxin A Decreases Acetylation of Tubulin, Leading to Microtubule Depolymerization through Activation of Histone Deacetylase 6, and This Mediates Acute Inflammation". *Journal of Biological Chemistry*. 285(43): 32888-32896

Naranatt, P., Krishnan, H., Smith, M., Chandran, B. (2005) "Kaposi's Sarcoma-Associated Herpesvirus Modulates Microtubule Dynamics via RhoA-GTP-Diaphanous 2 Signaling and Utilizes the Dynein Motors to Deliever Its DNA to the Nucleus". *Journal of Virology*. 79 (2): 1191-1206

Palazzo, A.F., Cook, T.A., Alberts, A.S., Gundersen, G.G.(2001) "mDia mediates Rho-regulated formation and orientation of stable microtubules". *Nature Cell Biology*. 3:723-729

Rajas, Ana M., Fuentes, G., Rausell, A., and Valenci, A.(2015) "The Ras Protein Superfamily: Evolutionary Tree and Role of Conserved Amino Acids." Journal of Cell Biology 192 (2012): 189-201

Riching, K.M., and Keely, P.J. (2015) "Rho family GTPases: Making it to the third dimension". *International Journal of Biochemistry and Cell Biology*. 59: 111-115

Westermann, S., and Weber, K. (2015) "Post Translational Modifications Regulate Microtubule Function." Nature 4 (2003): 938-47

Zilberman, Yuliya, Ballestrem, C., Carramusa, L., Mazitschek, R., Khochbin, S., and Bershadsky, A. (2015) "Regulation of Microtubule Dynamics by Inhibition of the Tubulin Deacetylase HDAC6." Journal of Cell Science 122 (2009): 3531-541

Schofield, A.V., Steel, R., and Bernard, R.(2012) “Rho-associated coil-coiled kinase (ROCK) protein controls microtubule dynamics in a novel signaling pathway that regulates cell migration.” Journal of Biological Chemistry 287 (2012): 43620-43629