THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

REGULATION OF NEURONAL POLARITY BY CHANGING ACTIN AND MICROTUBULE DYNAMICS

MENGYING QIN

Spring 2011

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biology
with honors in Biology

Reviewed and approved by the following:

Gong Chen
Associate Professor of Biology
Thesis Supervisor

Sarah M. Assmann
Waller Professor of Biology
Honors Adviser

*Signatures are on file in the Schreyer Honors College

Abstract

Neurons are remarkable in that their polarized morphology is essential for its function of unidirectional information passage. The polarity of a neuronal cell is marked by the presence of a single axon and multiple dendrites. Here, we report that cofilin and its upstream regulators LIM kinase (LIMK) and Slingshot (SSH) are critical for axonal formation. We studied the role of cofilin in axonal regulation by disrupting cofilin and its regulators during critical time periods of neuritogenesis and axogenesis in cortical neuron cultures. We found that loss of cofilin, LIMK or SSH greatly reduced the percentage of single axon neurons. Contrarily, overexpression of cofilin or LIMK did not affect axonal determination but the overexpression of SSH prevented the formation of axons in many cells. As cytoskeleton activity is known to be crucial to axon formation, we co-expressed cofilin shRNA with the cytoskeleton proteins actin and tubulin in an attempt to repair the deleterious effects of cofilin knockdown. It appears that actin overexpression alone induced multiple axons, and also rescued axon defects caused by cofilin shRNA. Similar phenomena were observed when actin was co-expressed with LIMK shRNA as well as with SSH shRNA. We found that defects from cofilin knockdown were highly correlated to the actin activity and less dependent on tubulin expression. Our results suggest that increased actin dynamics result in more axons, while decreased actin dynamics result in increased probability of no axons.

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Introduction

Neurons are polarized

There is a great diversity of nerve cell classes in the brain but neuronal morphology is readily distinguishable and highly specialized. Its form of multiple and elaborate dendritic trees and single long axon fits the function of one-way directed information flow by signal reception and transmission within a circuit. In addition to the well characterized functional and morphological differences known to exist between axon and dendrite, it has been found using in situ hybridization and immunocytochemistry techniques that mRNAs, organelles and other molecular differences exist between axon and dendrites (Caceres et al., 1986; Bruckenstein et al., 1990; Shi et al., 2003; Stiess and Bradke, 2011). Of great importance to axonal identification studies was the finding that MAP2 and tau proteins are segregated into dendritic and axonal domains (Kosik and Finch, 1987).

The exact mechanism by which the elegantly polarized neuron achieves its final form has been under intense study since the first established model system to derive primary culture of dissociated rodent hippocampal neurons (Dotti et al., 1988). This method revealed that cultured neurons went through a stereotyped sequence of events from development of short undifferentiated processes at several hours, to the rapid elongation of only one of those short processes to become axon.

Polarity Study Model Systems

Dissociated hippocampal culture is a commonly used model system to study neuron polarity. At rat embryonic day 18 (E18), hippocampal cells are isolated and plated at low density in culture which “resets” the neuron into a round ball (Kaech and Banker, 2006). Plated neurons

follow a consistent and predictable schedule of development. Stage one neurons are those immediately following dissociation when the neurons' morphology begins as a round and undifferentiated ball. Hours following plating, the neurons are at stage two and show extension of equal numbers of neurites. These neurites are actively competing to inhibit other neurites and giving self positive feedback signals for extension and growth. At stage three, one of the neurites is successful and elongates into an axon and signal the rest to become dendrites (Goslin and Banker, 1989). Polarity studies have since expanded to use not only hippocampal cells in the classic paradigm, but also other cell types. One study using cerebellar granule neurons revealed new data about cell-type specific polarization of bi-axonal parallel fiber (Powell et al., 1997).

Finally, some more recent models manipulate genes in the progenitor cells before polarization and forgo dissociated cultures in favor of visualizing cells in vivo (Hand et al., 2005; Calderon de Anda et al., 2008). One such study used pharmacological drugs, dominant negative mutants, as well as shRNA knockdown methods found that inhibition of CAM kinase I activity essential for single axonal development (Davare et al., 2009).

Role of Cytoskeleton

Cytoskeletal proteins are integral to the maintenance as well as establishment of not just neuronal cells, but other polarizing cells as well (Refer to Nature Mol Cell Bio Reviews: Li 2008). Actin filaments and microtubules are polarized in structure and they allow for rapid and long term conversion of signaling molecules into functional morphological modification of cell structure (Li and Gundersen, 2008). Studies using actin-destabilizing agents such as lactrunculin B and cytochalasin D have shown that remodeling the actin cytoskeleton plays a substantial role in regulating axon formation. Depolymerizing a single neurite in the unpolarized stage two neuron is sufficient to induce axonal formation in that neurite and application of lactrunculin-D

to 1 day old cells cause appearance of multiple fast growing processes containing membrane organelles characteristic of axons (Bradke and Dotti, 2000).

Tubulin works in parallel with actin to form axons and it was demonstrated that photoactivation of taxol to stabilize tubulin can confer axonal fate to a single dendrite (Witte et al., 2008). Similarly, the microtubule destabilizing chemical nocodazol reduces the formation of dendrites (Podkowa et al., 2010).

Severing the axon in a cultured neuron can result in microtubule dynamic change and subsequent reassignment of another neurite to the position, but it has been shown that regeneration of axon from dendrite is dependent on the distance of axotomy from the soma (Dotti and Banker, 1987; Goslin and Banker, 1989; Takahashi et al., 2007). Additionally, recent studies demonstrate that microtubule stabilization can even contribute to axon regeneration in mature neurons integrated in synaptic networks (Gomis-Ruth et al., 2008; Hellal et al., 2011). Pharmacological stabilization of microtubules was even able to facilitate the transformation of dendrites into multiple axons (Gomis-Ruth et al., 2008).

The effects of overexpression of cytoskeletal proteins on neuronal polarity have not been well characterized. This topic is of great interest to our study because of the actin severing actions of cofilin as well as the known implications of microtubule activity on neuronal polarity.

ADF/Cofilin

Actin dynamics are regulated by a variety of mechanisms from nucleation, branching and bundling proteins such as Arp2/3, formin, CaMKII and Ena/VASP (Rohatgi et al., 1999; Neidt et al., 2009; Ducka et al., 2010; Hansen and Mullins, 2010). Recent studies in axon regeneration in the CNS have found that actin polymerizing regulators downstream of Rho play a role in axonal

growth and that axonal growth was correlated with increased levels of cofilin (Ahmed et al., 2010). Actin severing protein such as those of the Actin Depolymerizing Factor (ADF) / cofilin family are highly conserved in all eukaryotes and strongly implicated in neuronal polarity (Lappalainen, 2007).

ADF/cofilin is the primary factor in dynamizing actin filaments by causing high turnover rates as well as severing filaments (Lappalainen, 2007). In polarized cell, and as especially characterized in fibroblast cells, cofilin is found at the leading edge to establish and maintain cell polarity (Bamburg and Bernstein, 2010). Cofilin is concentrated and highly active in axonal growth cones, and knocking down cofilin results in impaired axon formation (Garvalov et al., 2007). Additionally, overexpression of wildtype cofilin lead to increase in the length of the longest process suggesting that cofilin is not only necessary for axonal formation, but also promotes axonal growth.

LIM kinase (LIMK) and Slingshot (SSH)

Cofilin's role in determining neuronal polarity can be better understood when the activity of its major regulators are taken into account. LIM kinase (LIMK) and slingshot (SSH) have been found to colocalize with cofilin in the growth cones of hippocampal neurons (Soosairajah et al., 2005). This suggests that these two proteins actively regulate cofilin activity and should be carefully examined in the study of cofilin's role on neuronal polarity.

Cofilin is inhibited upon phosphorylation of a conserved N-terminal serine3 by LIM kinase (LIMK) and activated by dephosphorylation of the residue by Slingshot (SSH) (Huang et al., 2006). LIM kinase's two catalytic N-terminal domains employ distinct signal transduction pathways to regulate neurite extension. The PDZ domain inhibits neurite outgrowth induced by

ROCK and LIM 1/2 domain inhibits neurite outgrowth induced by either ROCK or NGF (Birkenfeld et al., 2001). It has been found that LIM kinase levels must be reduced for axon initiation (Tursun et al., 2005). However, the role of LIMK as a negative regulator of neurite extension is not yet clearly defined as other studies have demonstrated LIMK's ability in remodeling the cytoskeleton to encourage neurite formation (Lee-Hoeflich et al., 2004; Rosso et al., 2004; Tursun et al., 2005). SSH not only activates cofilin to induce motile growth cones and increase neurite extension rate, but it also antagonizes cofilin's inhibitor, LIMK (Endo et al., 2003). When either SSH1 or SSH2 isoform was knocked down, the percentage of cells with neurites decreased (Endo et al., 2007). A double knockdown of SSH1/2 decreased the mean neurite length as well.

The previous research shows the ability of cofilin's regulators to influence neurite growth and suggests that cofilin may also be able to influence axonal determination.

Findings

In this study, we examined the roles of cofilin, LIMK and SSH on neuronal polarity by using short hairpin RNA (shRNA) to silence their expression level in neurons. We further examined the possibility of cytoskeleton protein overexpression compensating for the loss of cofilin and its upstream regulators.

Knockdown of cofilin and its regulatory proteins resulted in increased numbers of cells with abnormal axonal formation as compared to the wild type. We further examined the potential rescue properties of overexpressing actin and tubulin in those cells. Our results indicate that actin overexpression may induce multiple axon formation. Additionally, increased pool of tubulin played a less potent role in axonal determination.

This study demonstrates that cofilin, its upstream regulators and its downstream effectors together play a crucial role in determining the number of axons generated per neuron.

Experimental Procedures: Materials and Methods

Neuronal Culture

Embryonic day 18, (E18) mouse cortical neurons were cultured as previously described (Deng et al., 2007). Low density neuronal culture of $2.4x10^4$ cells per cover slip was used.

Transfections

Calcium Phosphate Transfection of Hippocampal Neurons:

A modified version of (Jiang and Chen, 2006) was used on 1 DIV hippocampal neurons. The following changes were made:

- Only 0.5 μg DNA of each protein was used when during co-transfection with 1+ protein
- Total neuronal transfection medium in well was reduced to 500 μl before DNA mixture was added
- DNA mixture was incubated with coverslip for 8 minutes at room temperature
- Suspension solution was incubated with coverslip for 25 minutes at 5% CO_2
- 10% CO_2 medium was replaced with 5% CO_2 medium after 10 minutes or until no more precipitate could be visualized

Polyethyleneimine (PEI) mediated transfection of Human Embryonic Kidney 293T (HEK-293T) cells:

1 μg DNA was added to 4 μg PEI and 100 μl Opi-Mem. Mixture was incubated for 5 minutes at room temperature and then added drop wise to the well. Cells were incubated in 5% CO_2 incubator for 4 hours. After 4 hours, all the medium was replaced.

Immunoflourescence

3 days after transfection of neuronal culture, cells were washed in PBS, fixed in 4% paraformaldehyde in PBS for 40 minutes at room temperature, permeabilized for 3 minutes with 0.1% Triton-X in PBS, and blocked for 30 minutes with 5% NGS or NDS in PBS. Cells were incubated with primary antibody overnight at 4°C, washed with PBS, and incubated with secondary antibody for 40 minutes at room temperature. Cells were mounted onto slides with Invitrogen ProLong Gold antifade reagent with DAPI. The following primary antibodies were used: 1:300 Mouse-anti-Tau, 1:500 Polyclonal Rabbit-Anti-Map2, 1:2000 Chick-anti-GFP. The following secondary antibodies were used: 1:300 Invitrogen Alexa Fluor 647 Goat-anti-Mouse, 1:300 Invitrogen Alexa Fluor 546 Goat-anti-Rabbit, 1:300 Invitrogen Alexa Fluor 488 Goat-anti-Chick.

Quantification

Axons were identified using previously described methods where MAP2 marked dendritic processes and tau marked axons (Kosik and Finch, 1987). 8 hors after plating equal map tau was observed in processes. 16hrs after plating neuritogenesis took place.

Western Blot

72 hours after PEI transfection of HEK 293T cells, cultures were harvested with 200 µl lysate buffer (1 ml Lysate buffer: 1 ml IP lysis buffer, 5 µl phophatease inhibitor cocktail, 5 µl protease inhibitor cocktail, 10 µl PMSF) on ice. 20 µl LDS sample buffer was added to the cell solution which was then sonicated. Beta-mercatoethanol was added at 1:100 dilution and the sample was boiled at 97°C for 10 minutes. Samples were separated on 10% SDS gel. Amersham Hybond membrane was treated with methanol for 15 seconds, rinsed with ddH_2O for 3 minutes and soaked in 1x Transfer Buffer for 20 minutes. Protein was transferred from gel onto membrane at 97 V, 280 mA, for 1.5 hours. Membrane was washed with TBS and blocked with 5% nonfat milk for 1 hour at room temperature. The membrane was incubated overnight at 4°C with primary antibody, washed with TBS-T, followed by 1 hour incubation at room temperature with HRP conjugated secondary antibody. Protein bands were observed using 600 µl Luminata Forte Western HRP substrate. The Rabbit-anti-Cofilin antibody from Cytoskeleton was used.

Western Blot Band Analysis

Using Photoshop CS3, the images from the western blot film were first inverted. Then each band was selected using the lasso tool and the following histogram information was collected: mean (brightness) and pixel (size). The product of these two values was called the absolute intensity. Relative intensity was calculated by normalizing values to the eGFP control band's absolute intensity. Standard error bars were calculated using the equation Standard Error of Mean (SEM) = Standard deviation (SD) / √number of repeats (3). Significance was calculated with two-tailed distribution, type-3 2-sample unequal variance T-test.

Statistics

All experiments were replicated at least three separate times. Statistical comparisons were performed using Fisher's exact test using Statistical Analysis Software, SAS. * designates a p value of less than 5%, ** indicates p value of less than 1%.

Results

This study aimed to examine the effects of cofilin and its regulators on axon formation. Overexpression of cytoskeleton proteins was used in an attempt to rescue polarity defects. One day (1 DIV) after neurons were dissociated from embryonic day 18 (E18) mouse embryos and cultured at low density, the neurons were transfected with a combination of plasmids along with eGFP marker. At 4 DIV cells were double-stained for MAP2, which visualizes dendritic processes, and TAU to visualize axons. Transfected cells were visualized by its fluorescent eGFP signal. The axons of the transfected cells were identified by the presence of increased tau signal distal to the soma combined with lack of MAP2 expression. The axons are marked by an arrowhead. (Fig 1A). Cells expressing eGFP and successfully stained for tau and MAP2 were analyzed and categorized into either having no axon, one axon, or multiple axons. The following experiments aim to answer these questions: 1) What is the role of cofilin in neuronal polarity, 2) Do cofilin's upstream regulators similarly affect polarity, and 3) If polarity defects occur, is it possible to overcome these by addition of cytoskeleton proteins?

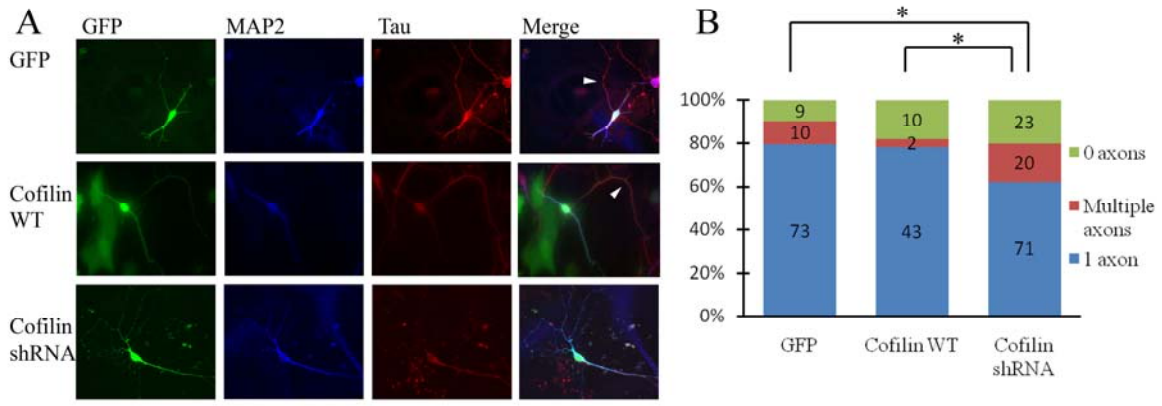

Figure 1. Cofilin Overexpression and Knockdown compared to eGFP control. (A) Immunocytochemistry images for cortical neurons transfected with eGFP alone or eGFP co-transfected with cofilin WT or cofilin shRNA. Arrowheads indicate axons characterized by high tau and low MAP2 signal. (B) Quantification of polarity defects in neurons expressing eGFP, cofilin or cofilin shRNA.

Cofilin KD, but not overexpression, results in polarity defect

To test the effects of cofilin's role in influencing axon formation, cofilin was overexpressed as well as knocked down in neurons at 1 DIV, which is a critical time point for axonal determination. Figure 1A shows a representative neuron from each experimental group. The first column is the eGFP signal indicating that the particular neuron has been transfected with each protein of interest. The second and third column marks the MAP2 and tau signal, representing dendrites and axon , respectively .

The control group, first row, represents neurons transfected with only eGFP at 1 DIV (Fig 1A). The image shown contains one axon, indicated by the arrowhead, which is the representative polarity profile of cells transfected with eGFP only. In Figure 1B, each column shown is the sum of at least three distinct batches of experiments. The control group shows that 80% of undisturbed neurons will grow only one single axon and the remaining 20% have similar amounts of multiple or zero axons (Fig 1B).

When the eGFP control group is compared with the findings from cofilin overexpression, no significant difference was found. However, when cofilin shRNA was transfected to knock down cofilin, the two groups were found to be significantly different. Neurons transfected with cofilin shRNA were 20% less likely to have a standard one-axon character. Instead, increased numbers were found to have multiple axons and even more were found to have no axons at all.

We find that overexpression of cofilin causes less changes in axonal formation than the knockdown of cofilin. Knockdown of cofilin decreases the percent of neurons with a single axon and increases the probability that a neuron has multiple or no axon, suggesting an important role of cofilin in maintaining normal neuronal polarity.

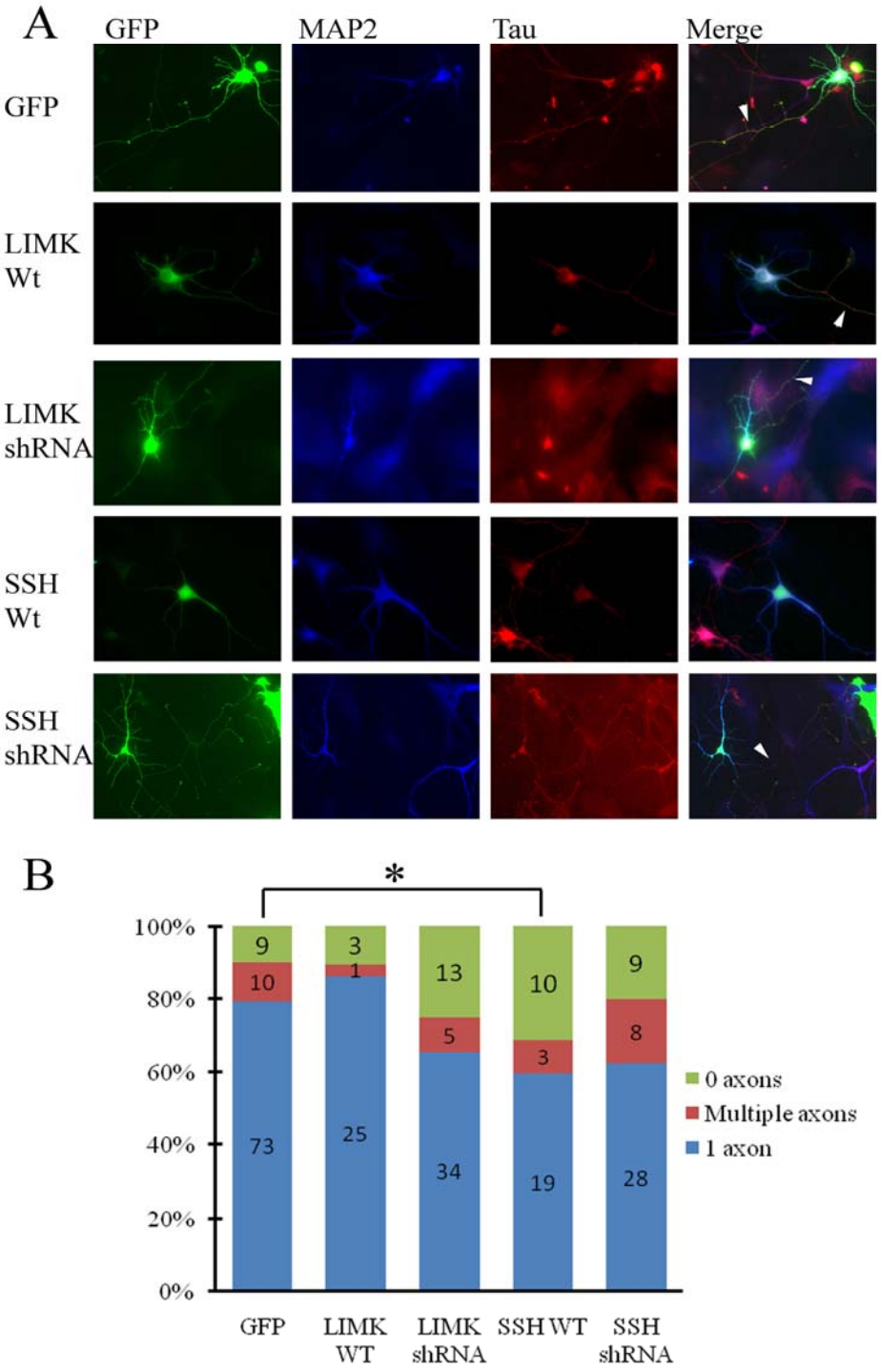

Figure 2. LIM kinase and Slingshot overexpression and knockdown. Immunocytochemistry images for cortical neurons transfected with eGFP alone or eGFP co-transfected with LIMK/SSH WT or LIMK/SSH shRNA. Arrowheads indicate axons characterized by high tau and low MAP2 signal. (B) Quantification of polarity defects.

SSH overexpression results in decreased axonal formation

To further test the effects of cofilin on neuronal polarity, cofilin's upstream regulators were studied. Cofilin's inhibitor, LIM kinase (LIMK), and its activator, Slingshot (SSH), were expressed in 1 DIV neurons (Fig 2A). The representative image for LIMK wildtype overexpression is a neuron with a single axon. The polarity profile of LIMK wildtype appears remarkably similar to that of GFP transfection in terms of percentage of single, multiple and no axon neurons. LIM kinase overexpression did not significantly alter the polarity results as compared to eGFP neurons. The representative image for neurons in which LIMK was knocked down is also a neuron with a single axon. LIMK shRNA expression was not significantly different ($p=0.06$); however, there was a greater than 10% chance of the possibility of neurons transfected with eGFP to have no axons than the neurons transfected with LIMK shRNA to not having any axon at all.

When SSH was overexpressed, there was a statistically significant increase in the number of neurons that lacked axons. The representative neuron shows heavy MAP2 staining in all neurites but no notable tau signal in any process (Fig. 2A). SSH appears to play a critical role in axonal formation, perhaps due to its dual action of not only dephosphorylating cofilin but also simultaneously inactivating LIMK (Endo, 2003).

The representative neurons from the SSH shRNA transfected group is a neuron with a single axon, just like the representative neuron from the eGFP transfected group. However, the polarity profile shows that when SSH shRNA is expressed, there is double the amount of multiple axons and double the amount of no axon compared to when eGFP is expressed in neurons. This indicates that while the majority of cells still grow one axon, the loss of SSH activity increases the probability for polarity defects.

When examining the effects of LIMK overexpression, we find that it resembles the trend in cofilin: protein knockdown is more potent than protein overexpression. In both cases the increase in levels of a specific protein shows less defects (multiple axons or no axon) when compared to the decrease in level of the protein. However, this trend is not continued in SSH knockdown and overexpression as both cases result in polarity defects.

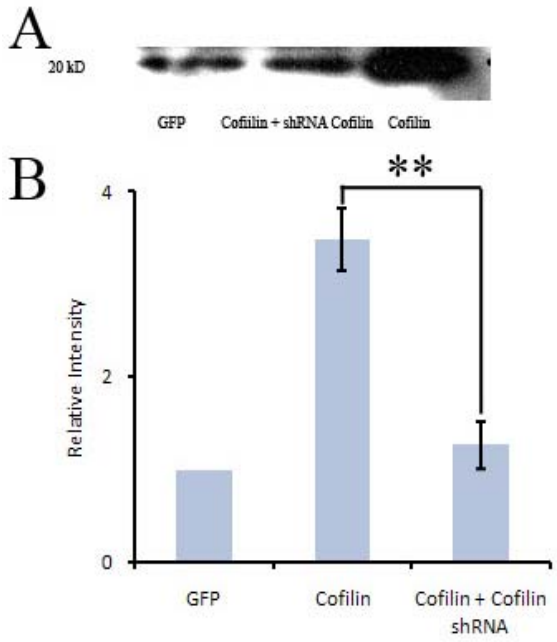

Figure 3. Cofilin shRNA is able to knock down cofilin after coexpression. (A) Bands at 20kD on membrane were stained for cofilin. (B) Ratio of band size and brightness to represent amount of protein present in GFP, cofilin, and cofilin + cofilin shRNA groups. (p=0.0078)

Cofilin shRNA is an effective knockdown tool

The previous and following experiments are highly dependent on the efficiency of shRNA to knock down target molecules. Human embryonic kidney 293T (HEK) cells were used to collectsufficient protein for western blot analysis. The total amount of cofilin from cofilin transfected cells was compared against total amount of cofilin from cofilin + cofilin shRNA transfectioned cells in order to examine the efficiency of the shRNA (Fig3).

HEK cells were transfected at high efficiency with PEI method, and the plasmids were allowed to express in cells for 72 hours. As described in the methods, western blot protocols were carried out to stain for cofilin expression, and the intensity and size of band were calculated and normalized to the eGFP control group. The first group transfected with only eGFP demonstrates the endogenous level of cofilin found in HEK cells. The second group, addition of cofilin, shows the significant increase in cofilin levels to more than triple the control levels. The third group is co-transfected with cofilin and cofilin shRNA. In the third group, the band size and intensity relating to the amount of cofilin expressed is slightly more, but not to a statistically significant degree, than eGFP.

We found that the shRNA used was able to effectively knock down the expression level of cofilin.

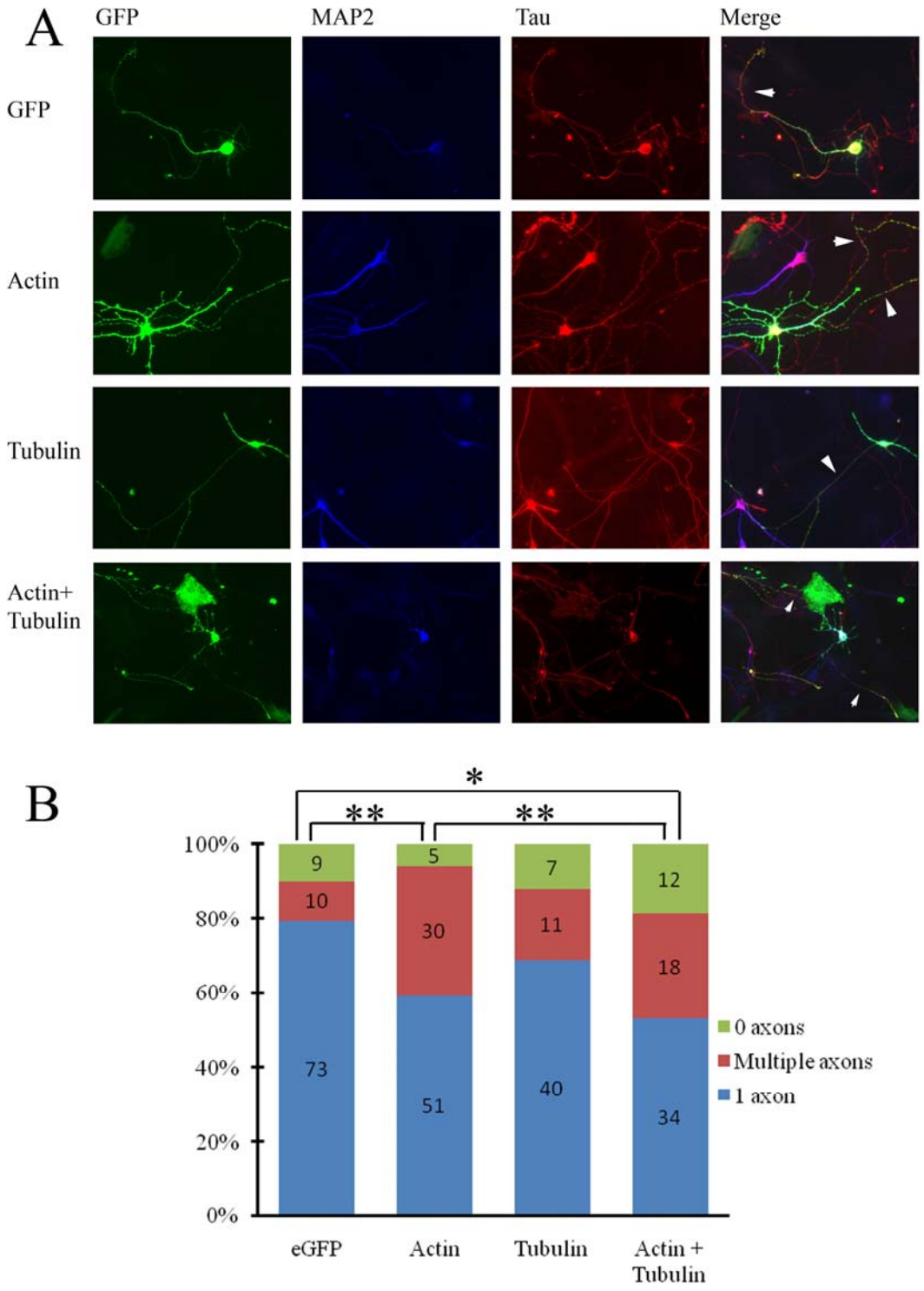

Figure 4. **Polarity of neurons overexpressed with cytoskeleton actin and tubulin.** (A) Immunocytochemistry images for cortical neurons transfected with eGFP alone or eGFP co-transfected with actin, tubulin or actin+tubulin. Arrowheads indicate axons characterized by high

tau and low MAP2 signal. (B) Polarity defect analysis of overexpression of cytoskeleton proteins compared to eGFP.

Cytoskeleton protein imbalance influences axonal formation in neurons

Once the efficacy of shRNA was confirmed, the next sets of experiments involved the use of actin and tubulin to rescue defects found in shRNA cofilin, LIMK and SSH neurons. This experiment examines the effects of actin and tubulin alone as a control for future experiments (Fig 4). Interestingly, overexpression of actin in 1DIV neurons showed significant ($P=5x10^{-4}$) increases in multiple axons coupled with a small decrease in rates of no axons. When actin was co-transfected with tubulin, there was again an increase in the occurrence of multiple axons, but more cells with zero axon than for the actin alone transfection, causing the actin and actin+tubulin groups to be different from each other. It appears that actin is a powerful player in axonal formation. Tubulin expression alone did not demonstrate significant difference from the normal control.

Actin is influential in affecting increased axogenesis. Because increased pool of actin increases the available monomers to participate in actin dynamics, there may be a correlation between actin dynamics and axon formation.

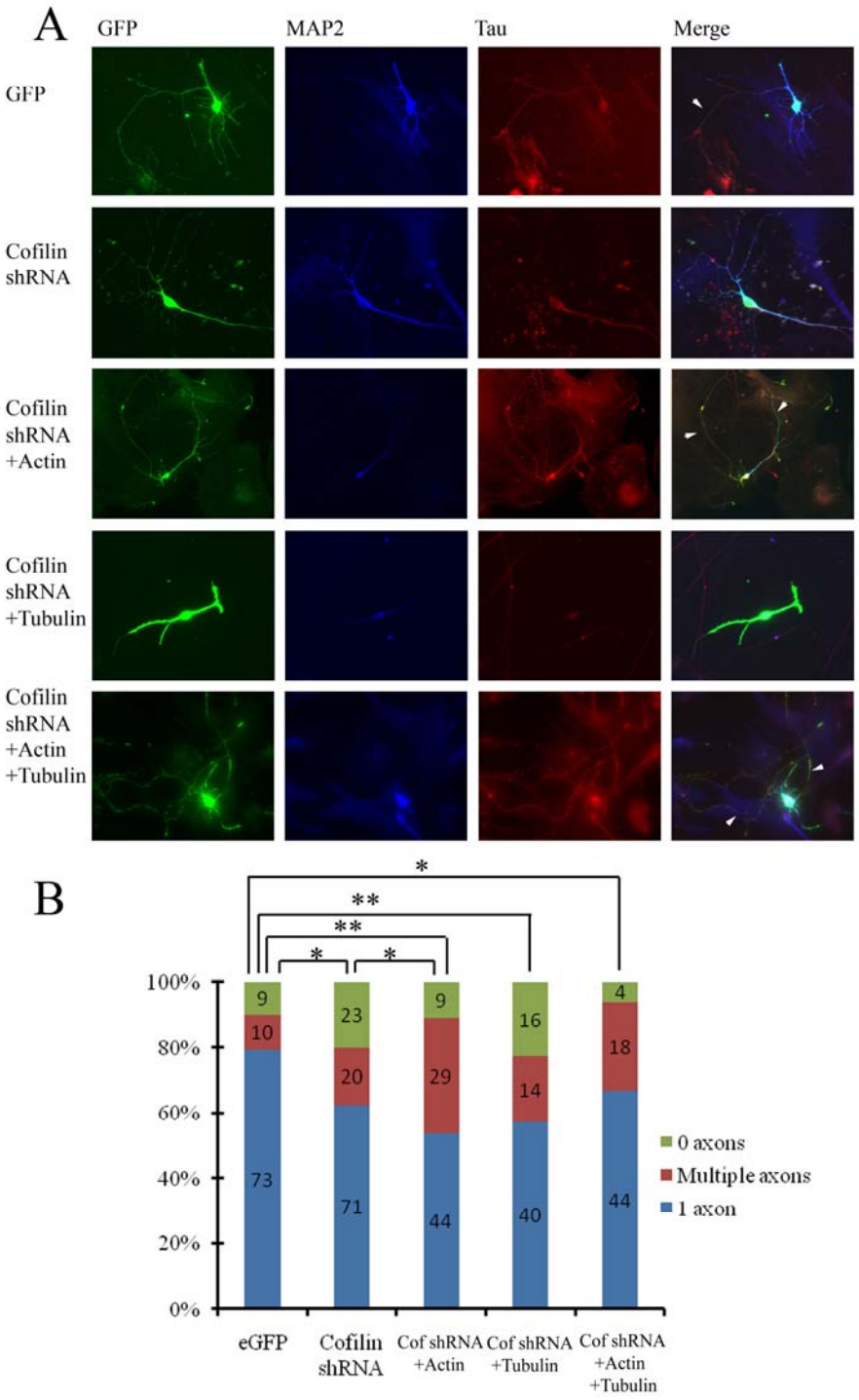

Figure 5. Polarity of neurons co-transfected with cofilin shRNA and cytoskeleton proteins. **(**A) Immunocytochemistry images for cortical neurons transfected with eGFP alone, cofilin shRNA alone, or cofilin shRNA plus actin, tubulin or actin+tubulin. Arrowheads indicate axons with high tau and low MAP2 signal. (B) Polarity defect analysis.

Cofilin shRNA + Actin neurons exhibit multiple axons

Actin overexpression has been found to increase the occurrence of multiple axons in neurons when compared to eGFP control groups. Tubulin overexpression has not demonstrated noticeable effect on neurons' polarity profiles. However, previous studies in the Chen Lab have suggested that cytoskeleton protein overexpression in conjunction with cofilin shRNA has potential to repair some polarity defects. Thus, further studies were done to study the potential of actin and tubulin to rescue the polarity defects caused by cofilin knockdown.

When actin and cofilin shRNA were co-transfected, there was an increase in cells with multiple axons and a decrease in cells with no axons (Fig 5). The percentage of cells with the standard "1 axon" was decreased from 80% in eGFP to 55% in cofilin shRNA +actin group. While the no axon effect of cofilin shRNA appears to be rescued, actin did not return the number of neurons with a single axon to normal and instead, increased the numbers of multiple axons.

Neither cofilin shRNA + tubulin nor cofilin shRNA + Actin + tubulin group was able to restore the number of single axons back to eGFP levels. Cofilin shRNA + Actin + tubulin increased the amount of multiple axons similar to the results in actin and actin+tubulin control groups. Cofilin shRNA + tubulin showed increased levels of no axons.

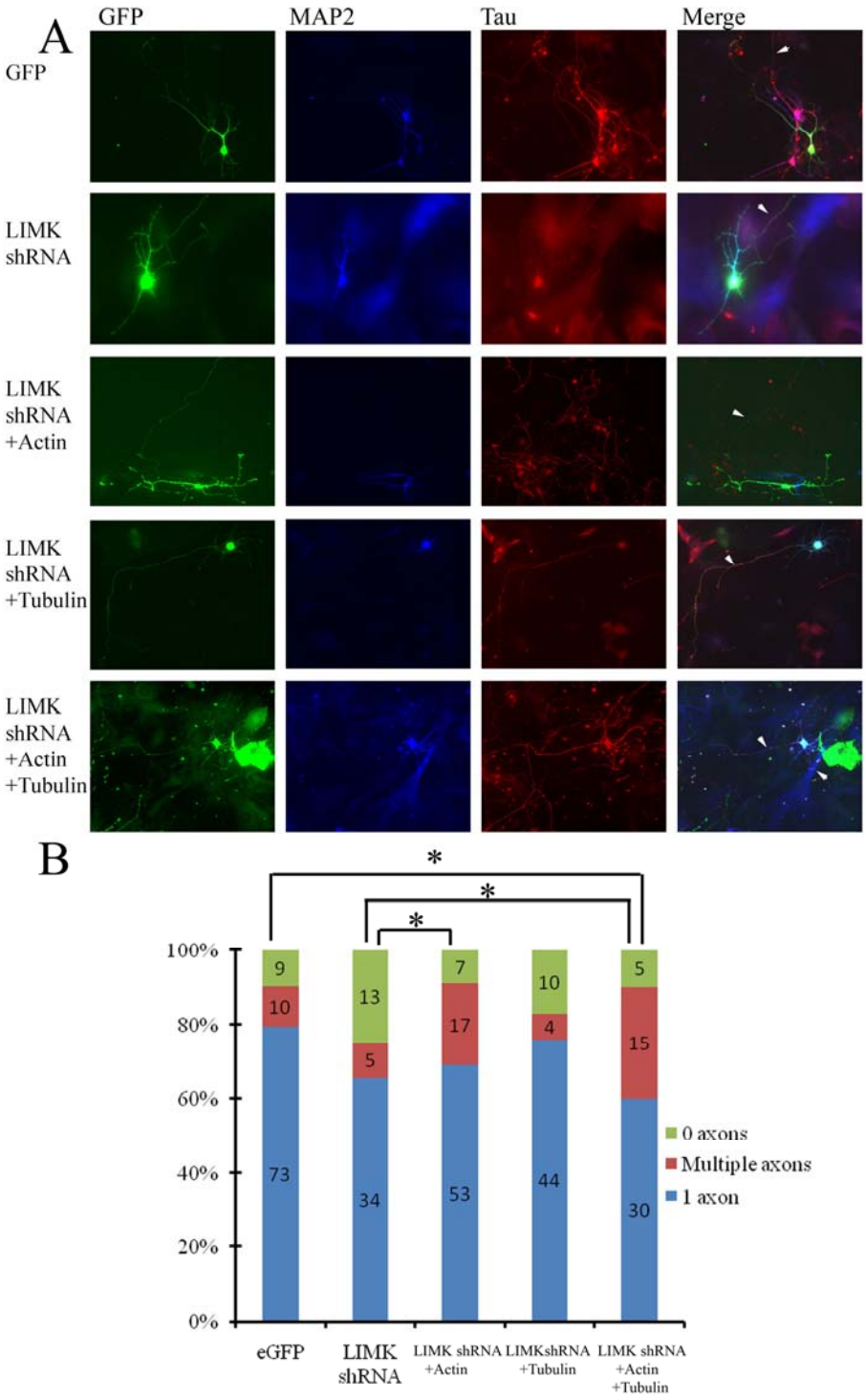

Figure 6. LIMK shRNA and rescue with cytoskeleton proteins. (A) Immunocytochemistry images for cortical neurons transfected with eGFP alone, LIMK shRNA alone or eGFP+LIMK co-transfected with actin, tubulin or actin+tubulin. Arrowheads indicate axons characterized by high tau and low MAP2 signal. (B) Polarity defect analysis.

LIM kinase shRNA neurons also show changes in the presence of excess actin

Addition of actin and tubulin appeared to show some amelioration to the deleterious effects of cofilin KD. Thus, we applied the same method to test LIM kinase shRNA and its interactions with actin and tubulin. It was found that LIMK shRNA did not show significant difference from eGFP group ($p=0.06$). When actin was expressed in conjunction with shRNA LIMK, there was no significant difference with eGFP group either ($p=0.13$), but it differed from LIMK shRNA alone by doubling the amount of neurons with multiple axons ($p=0.02$). Following the previously observed trend, actin does not increase the percentage of single axons. Even though the polarity profile shows decreased levels of neurons with single axon, the addition of actin still increased the level of polarized cells, with at least one axon, closer to control levels.

Also following the previously observed pattern, addition of tubulin to shRNA LIMK did not result in a statistically significant difference ($p=0.49$) but addition of tubulin and actin increased the amount of multiple axons.

Again, actin is able to rescue the polarity loss from LIMK shRNA but appears to overcompensate by increasing the number of axons. The data continues to suggest that actin plays a critical role in axon formation. Though tubulin's effect remains less pronounced, when co-transfected with actin, there is even more increased percentage of multiple axon.

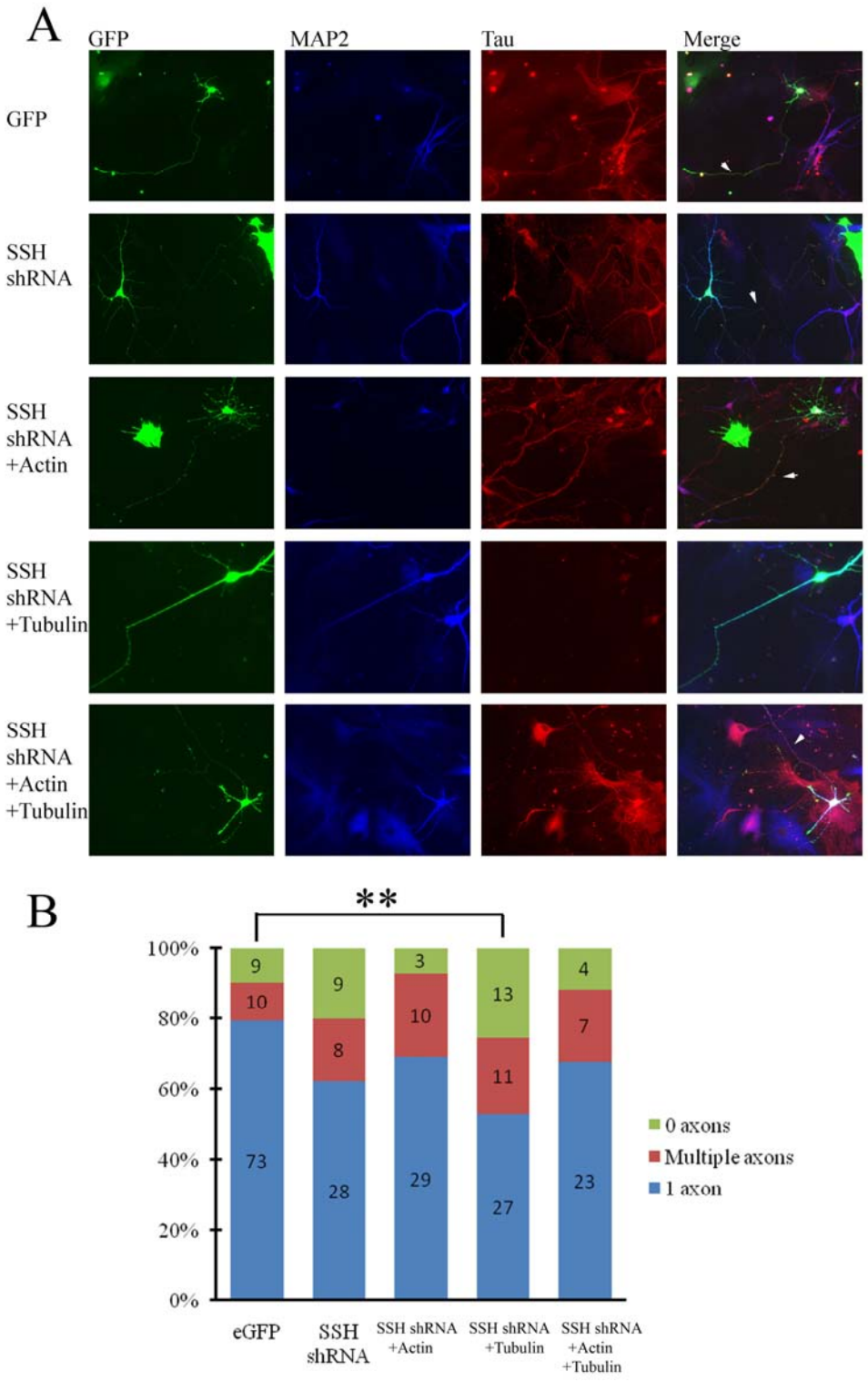

Figure 7. SSH shRNA and cytoskeleton's effects on polarity. (A) Immunocytochemistry images for cortical neurons transfected with eGFP alone, SSH shRNA alone or SSHshRNA + eGFP co-transfected with actin, tubulin or actin+tubulin. Arrowheads indicate axons characterized by high tau and low MAP2 signal. (B) Polarity defect analysis.

Slingshot shRNA combined with tubulin overexpression decreases axon formation

Because SSH is responsible for activating cofilin by dephosphorylation, the effects of SSH shRNA and actin and tubulin's rescue effects were examined to better understand the role of cofilin in cell polarity. Interestingly, in this set of data, the only combination that showed significant difference was between eGFP and SSH shRNA+tubulin ($p=0.004$). Addition of tubulin to SSH shRNA caused increased amounts of neurons possessing no axons and to a smaller degree, increased amounts of neurons possessing multiple axons.

Though SSH differed from cofilin and LIMK in that SSH was the only protein to have defects in both overexpression and knockdown (Fig 1 and Fig 2), SSH shRNA resembles the trend seen in both LIMK shRNA and cofilin shRNA in its response to increased levels of actin and tubulin. Addition of tubulin does not significantly change the effects of shRNA SSH and addition of actin returns the level of neurons that are polarized, that contain at least one neuron, back to levels found in the control group.

Discussion

Cortical neurons were used to examine cofilin and its regulatory proteins' effects on polarity. Control neurons were transfected with eGFP at 1DIV, the time that neurite formation is first initiated (Dotti et al., 1988; Buchser et al., 2010). Axons were identified as processes possessing tau and lacking MAP2 immunocytochemical signals after fixing and staining (Baas et al., 1991).

Cofilin/ADF is known to both depolymerize filamentous actin as well as to form a cofilamentous structure with actin (Lappalainen, 2007). Its ubiquity derives from its implicated participation in multiple aspects of cell function and thus, it is especially relevant to the study of cell polarization (Lappalainen, 2007). Increased cofilin activity has been implicated in the formation of axons (Barnes and Polleux, 2009). Overexpression of ADF/Cofilin directly promotes neurite extension and outgrowth (Meberg and Bamburg, 2000).

While it is known that an increase in cofilin affects neurites, it does not necessarily follow that axons are affected in the same way. In this experiment, the cofilin overexpression data which were prepared by a previous student does not demonstrate significant difference or increased axonogenesis. Perhaps overexpression of cofilin did not change the amount of active cofilin in the cell, or there may not have been an increase in overall severing activity. While there was not increase in axons, the change in neurite formation was not confirmed. Furthermore, the overexpression phenotype may be limited in its relevance as the activation of the pathway may be at a location not normally involved with axon specification and shRNA technology or gene knockout may provide a more accurate role of cofilin (Barnes and Polleux, 2009).

Cofilin knockdown was used to further study the effects of cofilin on axon formation. Significant difference in neuronal polarity was observed when cofilin is knocked down with shRNA. There is an increase in the number of neurons exhibiting zero neurons and to a lesser degree, an increase in the number of neurons with multiple axons. The knockdown of cofilin appears to severely disrupt normal axon determination or formation. Interestingly, the disrupted neuron has a nearly equal occurrence of having either none or multiple axons suggesting a complex mechanism.

To further understand cofilin's activity in axonogenesis, LIM kinase's effect on polarity was examined. The LIM kinase family is one of only two families, the other being TESK, with the ability to dephosphorylate cofilin (Huang et al., 2006). In this experiment, while neither LIMK overexpression or knock down showed statistically significant difference from eGFP, there appears to be a reduced number of single axon neurons when LIMK is knocked down. To some extent, loss of LIMK activity compromises the polarity of the cell.

Slingshot is one of only two known cofilin-specific phosphotases, each of which possesses an independent regulatory mechanism for cofilin activation (Huang et al., 2006). Slingshot not only activates cofilin, but works simultaneously to repress cofilin's inactivator LIM kinase. SSH overexpression has been demonstrated to induce both motile growth cones as well as increased neurite formation (Endo et al., 2003). In this experiment, overexpression did not increase the number of axons. However, there is a significant increase in the occurrence of neurons without any axons.

It has been shown in *Drosophila* that loss of SSH activity through mutation significantly elevates level of F actin in addition to phosphorylated cofilin (Niwa et al., 2002). In this

experiment, SSH shRNA does not demonstrate significant difference from eGFP control but it is seen that there is a decrease in number of neurons with a single axon.

The cofilin shRNA and LIM kinase shRNA have been previously shown to be effective. Future experiments will be conducted to confirm SSH shRNA's silencing efficiency. Unlike LIMK plasmid, the SSH plasmid used in these experiments lacks a fluorescent GFP signal. It also lacks any other tag that allows it to be readily visualized through western blot procedures. Therefore an indirect method involving prior knowledge of SSH interaction with cofilin and rods must be used. Cofilin overexpression is known to increase cofilin rod formation and cofilin + SSH will increase cofilin rod formation as well. If SSH shRNA is able to silence SSH, then cofilin+SSH+SSH shRNA will have decreased cofilin rods compared to cofilin+SSH overexpression.

The effect of cofilin's substrate, actin, on neuronal polarity was studied as a control for further experiments. The close relationship between cofilin and actin suggests that in the absence of cofilin severing, overexpression of actin might increase the actin activity necessary for axonal generation. Actin is a major component of the cytoskeleton and is highly dynamics; in eukaryotic cells, actin reorganization occurs with the aid of actin polymerizing and severing factors at the growth cone's periphery (Jiang et al., 2005). The polymerizing and depolymerizing activity of actin accounts for 50% of neuronal energy expenditure (Bernstein and Bamburg, 2003). Actin overexpression showed statistically significant different results from eGFP controls in that a large increase of multiple neurites was observed. It is hypothesized that increasing the pool of monomers available will cause an increase in actin and microtubule dynamics.

The increase in actin activity following transfection with actin follows previously observed phenomena. It has been shown that disruption of normal polarity processes can result in

neurons exhibiting multiple axons or axon-like neurites (Inagaki et al., 2001). Treatment of actin destabilizers in stage 2 neurons resulted in formation of multiple neurons (Bradke and Dotti, 2000). Actin filament depletion resulted in the formation of multiple axons in not only young neurons but as well in stage 4 neurons. In stage 4 neurons with established polarity, actin destabilization resulted in multiple neurites with MAP2 present proximally and tau distally suggesting that dendrites are plastic and able to gain axonal properties (Bradke and Dotti, 2000).

Tubulin is another essential building block of the cytoskeleton. When polymerized, tubulin forms microtubules to lay the tracks by which kinesin motors transport cargo necessary to establish axonal specification (Jacobson et al., 2006). Microtubules are prominent in the central region of the growth cone and its' dynamic regulation at the tip of axonal growth cones have been implicated in axonal projection and guidance. When knockdown of an upstream protein causes microtubule to be destabilized, the axon branching rate, but not growth rate, was found to be increased in chick retinal neurons (Shintani et al., 2009).

The balance of microtubule dynamics is critical to neuronal differentiation. Moderate or extreme microtubule destabilization can lead to increased neurite growth and induce multiple axon formation. Extreme microtubule stabilization is able to block axonal growth (Witte et al., 2008). Microtubule stabilization by taxol treatment induced microtubule bundling in the cell body but did not induce axonal processes (Knops et al., 1991). A modulator of tubulin and microtubule dynamics, Kidins220/ARM, has also been previously known to regulate neuronal morphogenesis (Higuero et al., 2009).

In this experiment, tubulin overexpression did not differ statistically from eGFP but it can be seen that in each instance where either actin, microtubule or both cytoskeletal proteins were overexpressed, there was decrease in the number of single axon neurons and an increase in the

number of multiple axon neurons. One possibility is that in times of actin activity stagnation, accumulation of F-actin in the growth cones prevents microtubules passage to extend the neurite.

Axons branch during path finding and growth, but once established, stability and maintenance are the main focus. On the contrary, dendrites retain the capacity to grow and branch indefinitely. This is in part accounted for by the difference in microtubule organization between axons and dendrites; dynamic tyr-tubulin is distributed throughout dendrite but concentrated in only the axon's distal growing end (Kollins et al., 2009).

As rescue proteins for cofilin knockdown, neither actin nor tubulin demonstrated an ability to return polarity of cofilin shRNA to normal eGFP levels. However, overexpression of actin alone or actin and tubulin was sufficient to return the number of neurons with polarity (at least one axon) back to eGFP levels. Stable expression of tau in neuroblastoma cells induced multiple neurites in 2 hours though at 4 hours the cells began to accumulate phosphorylated tau as well as increase Capase 3 activity (Yoshizaki et al., 2004). Although in our experiment, addition of tubulin alone did not increase number of neurons with polarity so it appears that actin is the agent that rescues cofilin knockdown cells from losing polarity.

It has been shown that in the absence of cofilin severing activity, tested with both LIMK overexpression as well as cofilin siRNA knockdown, there was a reduced pool of actin monomers and rate of actin polymerization was reduced especially at the cell periphery (Kiuchi et al., 2007). The LIM kinases promote actin stability via cofilin inactivation and their overexpression has been shown to promote the accumulation of F-actin (Toshima et al., 2001). ADF silencing was also found to lead to prevention of phosphorylated MAP reorganization (Whiteman et al., 2009). Additionally, overexpression of cofilin showed increase in neurite

length, supporting the idea that axonogenesis and growth cone differentiation require actin turnover with regulation by cofilin (Meberg and Bamburg, 2000).

In neurons with SSH knockdown, actin and actin+tubulin showed small but non-significant increase in number of cells with polarity. This trend is similar to that seen in cofilin knockdown. However, interestingly, when tubulin was overexpressed in cells with SSH shRNA, there was a dramatic increase in both cells with no polarity and cells with multiple axons. It is known that when SSH, when downregulated with small RNA interference, will be accompanied by reduced synaptic plasticity as well (Yuen et al., 2010).

Following the trend seen in previous experiments, the addition of actin to LIMK shRNA neurons decreased the incidence of neurons lacking polarity while increasing the number of neurons with multiple axons. When tubulin was overexpressed in the LIMK shRNA cells, no increase in axons was observed. LIMK is known to regulate microtubule stability by phosphorylating p25, a tubulin polymerization promoting protein (Gorovoy et al., 2005). LIMK is implicated in normal microtubule function and LIM kinase downregulation with siRNA magnified the sensitivity of cells to microtubule destabilizing agents vincristin and vinblastine but not to microtubule stabilizing agents. In siRNA LIMK knockdown cells, phosphorylated cofilin was increased and loss of the F-actin network was observed, suggesting that LIMK KD increased the resistance to the microtubule destabilizers (Po'uha et al., 2009). LIM kinases, downstream effectors of Rho GTPase, are also shown to interact with BMP to directly remodel actin cytoskeleton and induce dendritogensis (Lee-Hoeflich et al., 2004). Rho GTPases lie upstream of cofilin and it has been shown that RhoA inhibits neurite extension while Cdc42 and Rac1 promote neuritogensis (Lee-Hoeflich et al., 2004).

The present study identified axons using immunocytochemistry techniques to stain for MAP and tau. Another method of identifying axons would be to visualize axon specific markers such as AnkyrinG found on the axon initial segment. While immunocytochemistry is a valuable tool for identification of axons, further study utilizing electrophysiology is necessary to determine the functionality of these axons.

Cofilin's effect on neurite formation has been characterized but cofilin's effect on axon formation has not. This study utilized overexpression and knockdown of cofilin to better understand the role of cofilin in axonal determination. The absence of cofilin and its regulators, SSH and LIMK, are deleterious to the formation of polarized neurons. Overexpression of cofilin or LIMK does not strongly influence polarity. The presence of excess actin in neurons during the period of neurite differentiation plays an influential role in influencing the creation of multiple axons. Tubulin does not strongly influence polarity. We believe that the activities of cofilin, LIMK and SSH during formation of axons is highly influenced by the dynamic activity of actin.

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Yuen EY, Liu W, Kafri T, van Praag H, Yan Z (2010) Regulation of AMPA receptor channels and synaptic plasticity by cofilin phosphatase Slingshot in cortical neurons. J Physiol 588:2361-2371.

Academic Vita of Mengying Qin

Mengying Qin
135 Spring Wood Drive, Allentown, PA, 18104
Mwq5004@psu.edu
Major in Biology, neuroscience focus
Honors in Biology

Thesis title: Regulation of Neuronal Polarity by Changing Actin and Microtubule Dynamics
Thesis Supervisor: Gong Chen

Work Experience

Pennsylvania State University Residence Life, University Park, PA
Summer 2010-Spring 2011
Resident Assistant
Manage floor of first year students
Supervisor: Katherine Ciullo

Pennsylvania State University Chemistry Department, University Park, PA
Spring 2011
Exam grader and proctor
Assist professor to grade and proctor Chem 202 examinations
Supervisor: Dr Christine D. Keating

Young Scholars Charter School of Pennsylvania, State College, Pennsylvania
Summer 2010
ESL teacher assistant
Assist with summer mathematics and English program for ESL students
Supervisor: Helena Khan

Grants Received

Schreyer Honors College Travel Grant - 2009
Eberly College of Science Travel Grant for exchange with Science Partner Institution (University of Sussex)- 2009
Schreyer Summer Research Grant -2010

Awards

Braddock Scholarship - 2007-2011
Schreyer Honors College Academic Excellence Scholarship – 2007-2011
Humanitarian Engineering and Social Entrepreneurship Change Agent Scholarship 2011

Research Skills

Neuronal transfection using calcium phosphate method
HEK cell transfections using Polyethyleneimine mediated method
Immunocytochemistry
Fluorescent Imaging
Western Blot Procedure

Photoshop to manipulate images and analyze data

Publications

Sun C, Qin R, Chen G et al. (2011) Identification and functional characterization of rare mutations of the neuroligin-2 gene associated with schizophrenia. Currently under review.

Community Service Involvement

Unite for Sight Vice President- Penn State Chapter – 2009-2011
Mount Nittany Medical Center Volunteer- 2010
Mount Nittany Medical Center Paramedic Assistant / EMT-B – 2010-2011

International Education

University of Sussex, Brighton, UK - Spring 2009 Semester Abroad
Hearts for Hershey Mission Trip, Guayaquil, Ecuador – Fall 2010 observational study
Mashavu Core Team, Humanitarian Engineering and Social Entrepreneurship Venture in Kenya - 2011

Language Proficiency

Mandarin Chinese, French - Intermediate