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The actin cross-linking protein AFAP120 regulates axon elongation in a tyrosine phosphorylation-dependent manner

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Abstract

Growth cone guidance and axon elongation require the dynamic coordinated regulation of the actin cytoskeleton. As the growth cone moves, actin-dependent forces generate tension that enables protrusive activity in the periphery and drives growth cone translocation. This dynamic remodeling of the actin cytoskeleton in response to membrane tension requires activation of Src kinase. Although it has been proposed that these actin-dependent forces vary with the extent of actin cross-linking, the identity of the cross-linking protein(s) remains unknown. AFAP120 is a nervous system specific actin cross-linking protein that is regulated by Src kinase phosphorylation. Here, we report that AFAP120 is expressed and tyrosine phosphorylated in differentiating cerebellar granule cells, where it is enriched in the axon and growth cone. Over-expression of AFAP120 enhances neurite elongation in a tyrosine phosphorylation dependent manner. These findings suggest that AFAP120 may coordinate Src signaling with the dynamic changes in the actin cytoskeleton that drive growth cone motility and axon elongation.

Keywords

axon elongation; growth cone; Src kinase; actin cytoskeleton; AFAP

Introduction

In developing neurons, the elongation of axons is guided by the motility of the growth cone, a highly dynamic structure at the distal end of the axon. Growth cones sense and translate extracellular signals into directed migration and axon extension. This motile morphogenic process is driven by dynamic reorganization of the actin and microtubule cytoskeletons [4]. Guidance signals, whether soluble or substrate bound, can induce receptor clustering which may in turn induce local changes in membrane tension and trigger intracellular signaling cascades and cytoskeletal reorganization. In growth cones, Src activation in response to ligand-induced tension is essential for cytoskeletal reorganization preceding growth cone turning [14,27,28]. Although the mechanism of Src activation and its downstream targets have not been determined, electron microscopy studies of actin organization in the growth cone suggest that at least one actin cross-linking protein is involved [19,20,26].

The Actin Filament Associated Proteins of 110/120 kDa (AFAP110/120) have the molecular binding properties required to coordinate Src signaling with actin remodeling. AFAP110/120 (AFAPs) are multi-domain actin cross-linking proteins that are capable of oligomerizing and binding to Src and Protein kinase C (PKC, Fig 1A; [2]). AFAP110 is ubiquitously expressed, while alternative splicing of the AFAP gene produces AFAP120, which is expressed

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specifically in the nervous system [6]. In non-neuronal cells AFAP110 plays an important role in formation of actin stress fibers, focal adhesions [5] and podosomes (adhesive actin-based structures found in Src transformed cells [9]) and is required for mechanical stretch-induced activation of Src [12,21,22]; these functions are blocked by a mutation in the AFAP110 SH2-binding domain that inhibits Src binding and AFAP110 tyrosine phosphorylation [1,12].

AFAPs contain a single actin binding domain, so their ability to cross-link actin filaments depends on oligomerization [2]. Although phosphorylation is not required for AFAP binding to F-actin [25], AFAP oligomerization is regulated by Src-dependent tyrosine phosphorylation [24], so phosphorylation also regulates the ability of AFAPs to cross-link actin filaments.

Relatively little is known about the function of AFAPs in the nervous system. Staining of brain sections with an antiserum that recognizes both AFAPs indicates that AFAPs are widely expressed in the developing brain and cerebellum [3]. AFAP expression decreases in the adult brain, remaining high only in regions that undergo continuous adult neurogenesis [3]. These data suggest that AFAPs may play a role in differentiating neurons. In this report, we demonstrate for the first time that AFAPs are present in the growth cone and axon shaft of differentiating cerebellar granule neurons and that AFAP120 regulates axon extension in a tyrosine phosphorylation dependent manner. Together, these findings suggest that AFAP120 may be one of the actin cross-linking proteins that regulate growth cone actin dynamics in response to Src activity.

Materials and Methods

Expression vectors

Replication-deficient recombinant adenoviruses expressing AFAP120-WT or phosphomutant AFAP120-9F (all the tyrosine phosphorylation sites mutated to phenylalanine) were produced using the pAdTrack-CMV shuttle vector and the AdEasy system [13]. The pAdTrack-CMV shuttle vector expresses the gene of interest (e.g. AFAP120) under a CMV promoter with EGFP expressed under a second CMV promoter. Viruses were purified on a CsCl gradient and titered on 293HEK cells as previously described [26].

Immunoblot blots

Cultured cerebellar granule cells (see below) were lysed in 50 mM Tris, 1.0% NP-40 and 150 mM NaCl containing protease and phosphatase inhibitors. Protein concentration was determined using the BCA assay (Pierce) and 10 μg/lane of cerebellar neuronal lysate were separated on an 8% SDS-PAGE gel. For phosphatase assays, phosphatase inhibitors were omitted from the lysis buffer and 40 μg of lysate were mixed with 4 μg of λ-phosphatase (Upstate) and 5 mM DTT in phosphatase reaction buffer, then incubated at 37°C for 10 min. The reaction was stopped by addition of sample buffer and boiling for 5 min. Antibodies included anti-phosphotyrosine 4G10 (Upstate), anti-AFAP (F1), anti-phospho-AFAP (PY94-AFAP) and Horse radish peroxidase labeled secondary antisera (Sigma). Signal was developed with ECL reagent (Amersham).

Cerebellar granule cell cultures

For dissociated cerebellar cultures, cerebella from postnatal day 3–7 mice were dissected, the meningies removed and the tissue chopped into small pieces. After digestion in phosphate-buffered saline solution containing 0.125% (w/v) trypsin (Invitrogen) for 20 min at 37°C, the tissues were mechanically triturated by repeated passages through a polished Pasteur pipette in phosphate-buffered saline solution containing 0.05% (w/v) DNAse (Invitrogen). Dissociated neurons were resuspended in the Neurobasal medium with B-27 supplement (Invitrogen) and plated poly-D-lysine coated coverslips.

For aggregated granule cell cultures, dissociated cerebellar granule cells were isolated and purified from P5-6 mice as essentially as described [8,16]. Briefly, isolated cerebella were incubated in 0.15% Trypsin type XII-S (Sigma) and Ca2+-Mg2+-free PBS for 20 minutes, re-suspended in media containing 0.25% DNAse I and triturated with a fire-polished glass pipette. Cells were kept in BME media (Gibco) supplemented with 10% FBS, 10% horse serum, penicillin/streptomycin, 0.2 mM L- glutamine and 6 mM glucose. Granule cells were then isolated from the interface of a 35%–60% Percoll gradient and subsequently purified by sequential pre-plating on Petri dishes coated with 0.1 mg/ml Poly-D-lysine for 1 hour in media. 3×10^5 cells were then seeded in wells of 16-well Lab-Tek chamber slides (Nunc) and incubated in a humidified chamber at 37°C, 5% CO_2. After 1–3 hours, cells were infected with a multiplicity of infection (m.o.i.) of 20–40. Under these conditions, at least 50% of the cells were infected and no cyto-pathological effects due to virus infection were detected. 24 hours following infection, cell aggregates were collected by brief low-speed centrifugation and resuspended in the above media supplemented with B-27 (Invitrogen), 10 mM D-serine (Sigma), and 25 mM KCl. Aggregates were then transferred to glass coverslips coated with 50 mg/ml PDL (Sigma) and 25 μg/ml laminin (Invitrogen) and incubated for an additional 24 hours before processing for immunofluorescence.

Immunocytochemistry, image acquisition and reagents

Cells were fixed for 30 min at 37°C in 4% paraformaldehyde in PHEM buffer (60 mM PIPES pH 7.0, 25 mM HEPES pH 7.0, 10 mM EGTA, 2 mM $MgCl_2$) with 0.12M sucrose. After rinsing in PBS, coverslips were incubated in 10% fatty acid free bovine serum albumin (BSA) in PBS for 30 min, permeablized for 10 min in 0.2% triton/PBS, rinsed, and re-blocked in 10% BSA/PBS for 30 min. Alexa-labeled Phalloidin (Molecular Probes) was used to label F-actin and DAPI staining was used to visualize nuclei. Primary antibodies used included anti-AFAP [7], anti-BetaIII-tubulin (Promega) and anti-tyrosinated tubulin (Chemicon). Secondary antibodies were purchased from Jackson labs.

For granule cell aggregate axon imaging and analysis, aggregates were chosen if they were relatively symmetrical with aggregate diameters between 80–130 μm and their axons had minimal contact with axons from other aggregates. The centroid of each aggregate was determined in the DAPI channel image using the Openlab thresholding tool. Only regions of aggregates where all axons could be visualized in entirety were chosen for measurement. EGFP expressing cells were measured from the centroid to the distal tip of the axon and normalized to the mean length of control axons in the same experiment. Experiments were done in duplicate.

Results

AFAP120 expression and phosphorylation in developing neurons

To determine which AFAP isoforms are expressed in the developing cerebellum, lysates from cultured cerebellar granule cells were probed with an antiserum that recognizes both AFAPs. This analysis revealed that while both AFAP110 and AFAP120 were expressed, AFAP120 was relatively more abundant (Fig. 1B, left panel).

In non-neuronal cells, AFAP110 function is regulated by Src-dependent tyrosine phosphorylation [1,10]. In the presence of active Src, both AFAP110 and AFAP120 are highly phosphorylated on tyrosine 94 (Y94; [11] and XX and LML, data not shown). To determine whether AFAP110 and/or AFAP120 are phosphorylated, and thus potentially active, the cerebellar granule cell blots were probed with antiserum that specifically recognizes phosphorylated AFAP Y94. Surprisingly, only AFAP120 appeared to be tyrosine phosphorylated under these conditions (Fig. 1B, right panel). Treating the lysate with lambda

phosphatase to dephosphorylate all proteins abolished PY94 binding, demonstrating that the antiserum was phospho-specific (Fig. 1B, right panel). While we cannot exclude the possibility that AFAP110 is phosphorylated at levels below detection by this antiserum, probing the blot with the pan-phosphotyrosine antiserum 4G10 antiserum failed to detect a band at 110 kDa (data not shown). These findings suggest that AFAP120 may be the functionally relevant isoform in differentiating neurons.

AFAPs are enriched in the axon and growth cone of differentiating cerebellar granule cells

To determine the subcellular localization of AFAPs in neurons, cultures of primary cerebellar granule cells were fixed and double stained with antisera to AFAPs and neuronal specific βIII-tubulin. F-actin was labeled with phalloidin. After one day in culture, dissociated granule cell neurons often take on a migratory morphology, with a large growth cone at the end of the leading process/axon and a trailing process behind the cell body (Fig. 2A). AFAPs were detected in the cell body, axon shaft and growth cones of cerebellar granule cells (Fig. 2A). Within the growth cone, AFAPs appeared to be enriched in the transition domain, but were excluded from the peripheral domain (Fig. 1A, B, lower panels). Staining levels in the central domain were intermediate. This localization is consistent with AFAPs playing a role in growth cone motility and/or axon elongation, possibly by regulating actin structures in the transition domain.

AFAP120 tyrosine phosphorylation regulates axon extension

To determine if tyrosine phosphorylation of AFAP120 is important for neuronal differentiation, recombinant adenoviruses were used to express wild-type AFAP120 or non-phosphorylatable AFAP120-9F (in which all the Src tyrosine phosphorylation sites were mutated to phenylalanine) in cultured cerebellar granule cell aggregates. Under these conditions, the overexpressed protein is expected to oligomerize with the endogenous AFAPs and act as a dominant active or dominant negative. Infected cells were identified by expression of non-fused EGFP under a second promoter. To control for possible effects of the virus itself, all results were compared to cells infected with virus expressing EGFP alone.

Aggregates were kept in suspension for 24 hours, allowing time for expression of recombinant protein to reach moderate levels (as monitored by EGFP fluorescence), then plated on coverslips and allowed to differentiate for 24 hours. During this time, neurons extended long tubulin rich leading processes that will become axons (Fig. 3A) and underwent nucleokinesis (migration of the nuclei) toward the distal tip of the process (nuclei were visualized by DAPI staining, Fig. 3B), similar to cerebellar granule cells differentiating in vivo [15] and to dissociated granule cells in vitro (Fig. 2).

After 24 hours of differentiation, aggregates were fixed and the EGFP expressing neuronal processes were analyzed. Under these conditions, axons extended several hundred microns and were tipped by actin-rich growth cones (Fig. 3). Compared to the large growth cones on the dissociated granule cells (Fig. 2), growth cones in the aggregate cultures were relatively small and could be characterized as expanded or collapsed (red and green asterisks, respectively, in Fig. 3E–G). There was no significant difference in the percentage of expanded/collapsed growth cones between the control and treatment groups (data not shown).

In control neurons expressing EGFP alone, there was no relationship between the intensity of EGFP fluorescence and the level of AFAP staining (Fig. 3H). In contrast, in cells co-expressing EGFP and exogenous AFAP120-WT or-9F, the level of EGFP fluorescence was generally proportional to the level of AFAP staining (Fig 3 I–J), indicating that exogenous AFAP120-WT and-9F were present in both the axons and growth cones. AFAP staining patterns were similar in control and AFAP120 over expressing cells (Fig, 3 K–M), although exposure times

used for the over expressing cells were considerably shorter than those used for controls. Based on the F-actin staining, these growth cones did not appear to have well defined central, transition and peripheral domains; nonetheless, AFAP staining appeared to be less intense in the periphery of the growth cones (Fig. 3 K–M).

We next examined the effect of AFAP120 over expression on axon elongation. To control for experiment-to-experiment variation in the rate of process outgrowth, each measurement was normalized to the average length of processes expressing EGFP alone. Interestingly, over-expression of AFAP120-WT significantly enhanced axon length (Fig. 3N). In contrast, over-expression of non-phosphorylatable AFAP120-9F caused a slight, though statistically significant, reduction in process length compared to controls (Fig. 3N). No consistent changes in nucleokinesis were observed. These data indicated that AFAP120 plays a role in regulating granule cell axon elongation and that its function may be regulated by tyrosine phosphorylation.

Discussion

In this report, we tested the hypothesis that AFAP120 plays a role in regulating axon elongation. Growth cone guidance and axon elongation depend on the dynamic, coordinated regulation of the actin cytoskeleton [4] and many reports suggest that this coordinated regulation depends on activation of Src kinases [23,31]. AFAP120 is a neuronal-specific actin cross-linking protein that binds to and is regulated by Src [2]. We have shown that AFAP120 is expressed and tyrosine phosphorylated in differentiating neurons, and co-localizes with filamentous actin in the transition and central domains of neuronal growth cones (Fig. 2A, 3K–M), indicating that AFAP120 is properly positioned to alter growth cone actin dynamics.

Given its growth cone localization and its ability to cross-link actin and associate with active Src [2], we speculated that AFAP120 might regulate axon elongation. Indeed, we found that in cerebellar granule cell aggregates, over-expression of AFAP120-WT, but not unphosphorylatable AFAP120-9F, lead to an approximately 10% increase in axon length. If AFAP120-9F acts as a dominant negative, then the finding that AFAP120-9F caused only a slight decrease in axon length may indicate that expression levels of AFAP120-9F were not high enough to completely inhibit endogenous AFAP120. Alternatively, it is possible that AFAP120-9F was non-functional and the small decrease in axon length may not be physiologically relevant. In either event, these findings indicate that AFAP120 regulates axon elongation in a phosphorylation-dependent manner.

Interestingly, a similar magnitude of change in axon length occurs with Lis1 haploinsufficiency or inhibition of Rho Kinase in aggregate granule cell cultures [16]. Lis1 plays a critical role in neuronal migration and differentiation in vivo [29,30], suggesting that the magnitude of change in axon elongation in cultured granule cells is not directly correlated with the importance of these molecules in vivo. Supporting this conclusion, knockdown of Mena, an actin-binding protein found in the tips of growth cone filopodia, causes minimal changes in the rate of axon elongation in vitro [18], but has a dramatic effect on axon guidance in vivo [17]. We suspect that like other growth cone enriched actin-binding proteins, AFAP120 function in axon elongation may be secondary to a role in axon guidance. This is consistent with the observation that growth cone actin dynamics are important for guidance, while microtubule dynamics determine the rate of axon elongation [4].

In growth cones, Src activation in response to mechanical stretch (adhesion) leads to reorganization of actin structures in the transition domain, enabling microtubule advance into the periphery, followed by growth cone turning and axon elongation [27]. Given the ability of AFAP110 to regulate the formation of actin-based adhesive structures in other cell types, it is tempting to speculate that in neurons AFAP120 regulates axon elongation by coordinating the

Src signaling events that couple substrate adhesion to dynamic changes in the growth cone cytoskeleton.

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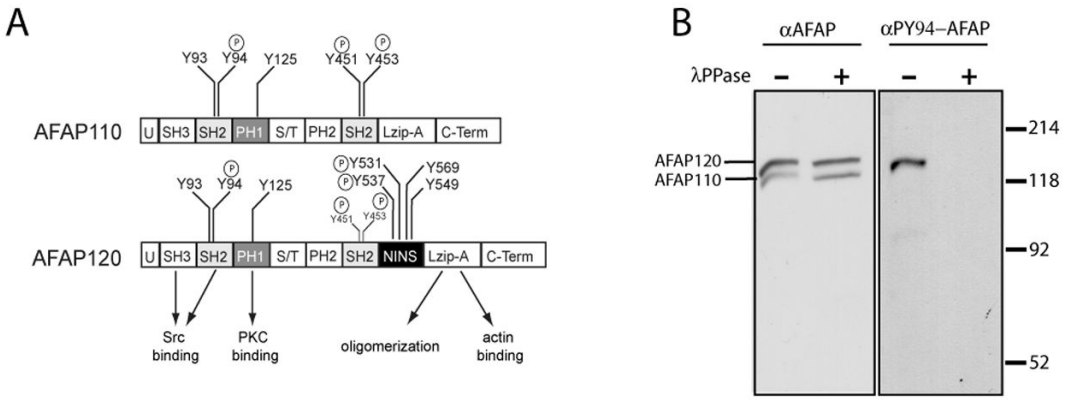

Figure 1. AFAP120 expression and tyrosine phosphorylation in developing neurons

(A) Domain structure of AFAP110 and AFAP120. AFAP110 and AFAP120 contain identical SH3-and SH2-binding domains, two pleckstrin homology domains (PH1 and PH2), a serine/threonine rich region (S/T) and a leucine zipper motif within which lies an actin-binding domain (Lzip-A). The SH2- and SH3-binding domains mediate AFAP interaction with Src, while the PH1 domains regulates AFAP association with PKC. AFAP120 contains a Neuronal Insert (NINS) that is generated by alternative splicing of an exon between the SH2 and Lzip domains. Positions of the potential Src kinase phosphorylated tyrosines (Y) are shown; major sites of Src dependent phosphorylation are indicated by an encircled 'P'. (B) Western blot analysis of AFAP expression in cerebellar granule cell cultures. Granule cell lysates were treated with (+) or without (−) λ-phosphatase, then immunoblotted and sequentially probed with PY94-AFAP antiserum that recognizes phosphorylated tyrosine 94 in both AFAP110 and AFAP120 and anti-AFAP antiserum that recognizes both AFAP110 and AFAP120. Positions of AFAP110 and AFAP110 are indicated on the left and molecular weight markers (kDa) are indicated on the right.

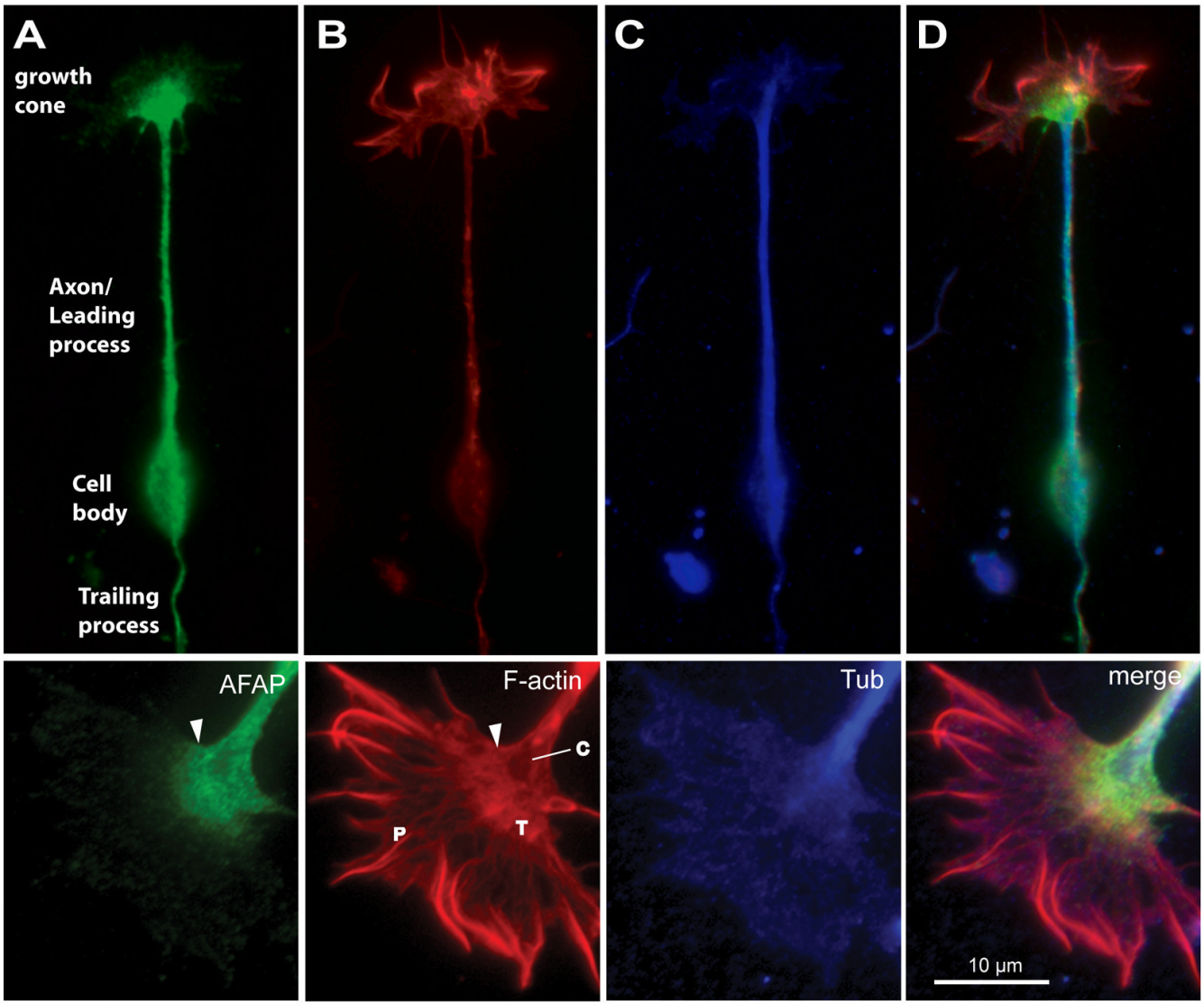

Figure 2. AFAP localization in cultured cerebellar granule neurons

Cultured cerebellar granule cells were fixed and stained with anti-AFAP (A, green), Alexa-phalloidin to detect F-actin (B, red) and anti-βIII-tubulin (C, blue). Merged images are shown in (D). High magnification of the growth cone region was shown in lower panels. (B) Lower panel; based on the organization of actin filaments, the growth cone can be partitioned into peripheral (P), transition (T), and central (C) domains. Arrowheads in (A) and (B) point to regions with AFAPs in transition domain.

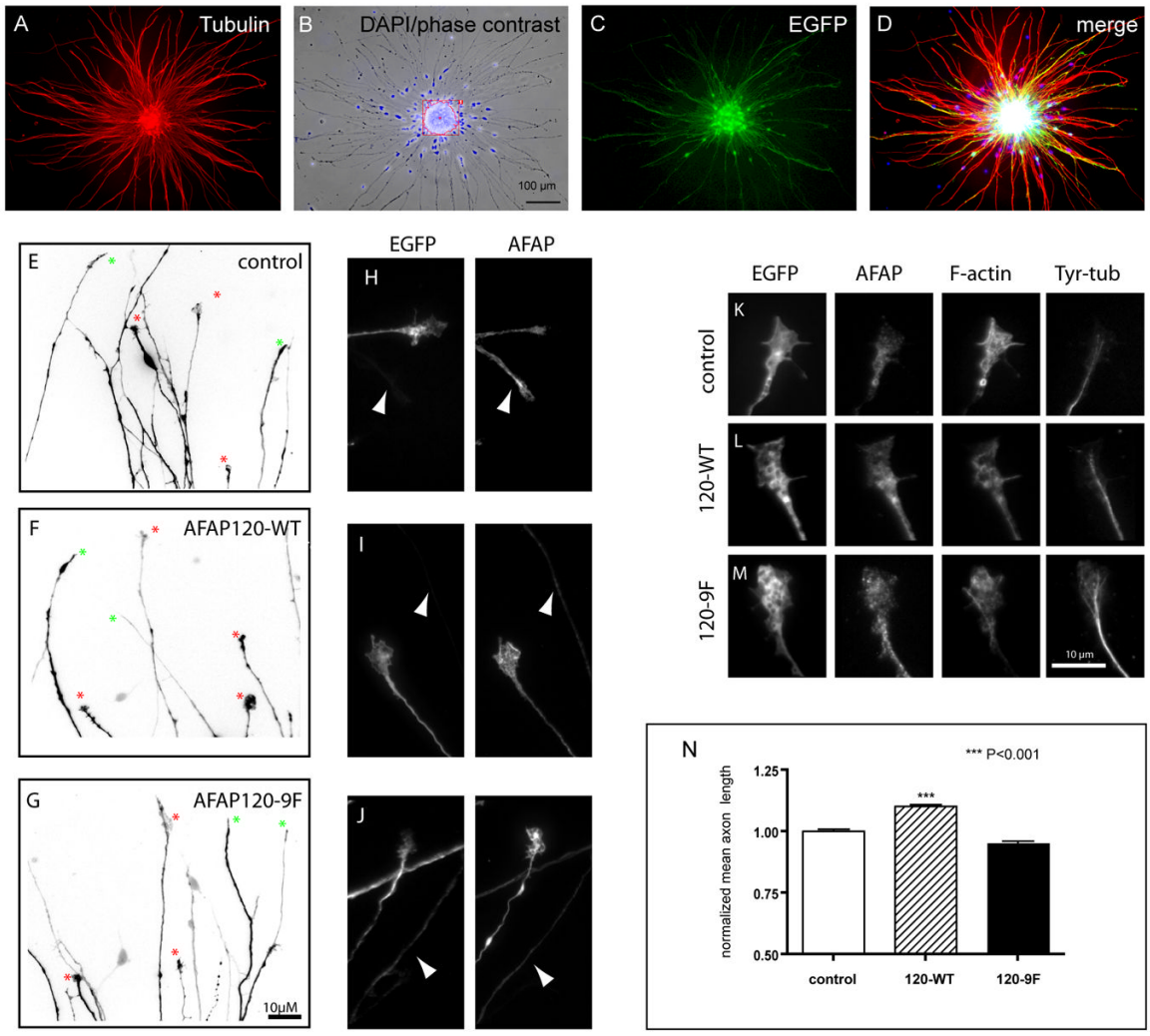

Figure 3. AFAP120 regulates cerebellar granule cell axon elongation in a phosphorylation-dependent manner

Axon length was measured in cerebellar granule cell aggregates infected with adenoviruses expressing EGFP alone (control), or co-expressing EGFP with either wild-type (120-WT) or phospho-mutant (120-9F) AFAP120. One day after plating, aggregates were fixed and immunostained with antibody to BetaIII-tubulin (A, red) and DAPI to detect nuclei (B, blue, shown superimposed on a phase contrast image). Infected cells were identified by EGFP expression (C, green). Merged images are shown in (D). Centroid position (center red point in B) is shown on the sample aggregate. Scale bar is 100 μm. (E-G) Examples of EGFP positive axons from control (E), AFAP120-WT (F) and AFAP120-9F (G) expressing aggregates. Growth cones were classified as expanded (5–10 μm diameter, red asterisks) or collapsed (<5 μm diameter, green asterisks). To enhance visibility, the signal has been inverted such that EGFP appears dark. Scale bar is 10 μm. (H-J) Relationship between EGFP and AFAP staining levels in controls (H), and in cells over-expressing AFAP120-WT (I) or AFAP120-9F (J). Arrowheads indicate axons with low EGFP expression. (K-M) Staining for AFAP, F-actin and tyrosinated tubulin in control (K), AFAP120-WT (L) and AFAP120-9F (M) growth cones. Scale bar is 10 μm. (N) Effect of over-expression of AFAP120-WT or-9F on axon length. Significance was determined by one way ANOVA with Bonferroni post hoc analysis and values are shown as mean±S.E.M. All three values are significantly different (P<0.001) from

each other. N ≥20 aggregates per treatment, with a total of ≥ 1000 axons measured per treatment.